METHOD FOR PREPARING A LIGAND PRESENTING ASSEMBLY (LPA), AN LPA, AND USES THEREOF

The present invention relates to a method for preparing a ligand presenting assembly (LPA), an LPA, an immunological composition and a vaccine. The invention further relates to a method for generating antibodies, a kit for use in diagnosis and use of an LPA for preparing a pharmaceutical composition.

BACKGROUND OF THE INVENTION

Peptides having an amino acid sequence corresponding to a part of the amino acid sequence of a protein or antigen are often used in immunoassays for screening for antibodies or preparation of vaccines.

The peptides or immunogenic compounds are often coupled to various carriers in order to enhance the immunogenic effect. One such system is multiple presentation of antigenic peptides, which has long been recognised as valuable means for amplifying peptide immunogens. Most commonly an antigen is attached to a natural carrier such as a protein, a carbohydrate, a lipid or a liposome to provide a vaccine component or a synthetic serodiagnostic.

There are, however, a number of problems with these products which may be related to the carriers. Since these are isolated from natural sources they may not be of uniform quality and may further contain harmful contaminants being themselves antigenic. When used for vaccines they may in particular result in fevers and tissue swelling. In attempts to overcome certain of these problems much research has in later years been focused on antigen presentation using purely synthetic carriers.

Dendritic polymers are a such class of polymers or synthetic proteins. Such polymers have been described widely in the literature. The polymers are often referred to as dendritic because of the similarity with a branched tree.

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A multimerisation known as multiple antigen peptide (MAP) using a branched architecture with a controlled and limited array of branches was described by Tam in PCT/US90/02039 (ref. 1).

10 MAPs have applications as synthetic vaccines and as diagnostic tools as well as other biochemical uses including immunoassays, serodiagnosis, epitope mapping, inhibitors, artificial proteins, intracellular delivery, affinity purification of antibodies and presentation of T-cell epitopes (ref. 2).

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All branched peptides are similar in that they branch from a core matrix or template which provide them with different dendrimeric properties (ref. 3, ref. 4). Most commonly, lysine is used as amino acid in the core matrix because it has two ends, the α- and ϵ-amino 20 groups; available for branching reactions.

Different types of branching have been reviewed recently (ref. 5, ref. 2) including cascade, pennant or radial type of branching arrangement (Fig. 1A, B and C in ref. 5). A stem peptide carrier 25 has been reported which is a short lipopeptide carrier, the stem peptide, which is useful for side-chain presentation of peptides (Fig. 2) (ref. 6).

The MAP's described above all have in common that an amino group 30 is the starting point for the antigen synthesis resulting in peptides which are attached to the core matrix by the C-terminal end and, thus, having a free N-terminal end.

The effectiveness of a B cell epitope on MAP to elicit humoral responses depends on the presence of a T cell recognition site located in the stretch of amino acids. While this is not always the case, chimeric B-T constructs have been made producing strongly enhanced immune responses. Such MAPs are known as diepitope MAPs. The effectiveness of diepitope MAPs over monoepitope MAP has among other been studied in synthetic vaccine models of the human immunodeficiency virus (HIV) type 1. There are many possible ways of arranging B and T cell epitopes on MAPs. Most commonly, they are linked in a tandem arrangement. High antibody titers were elicited by MAPs containing equimolar amounts of the T and B epitopes, while monoepitope MAPs and B cell epitope monomers showed no immunogenic effect in A/J mice used in the experiments, cf. ref. 5. MAPs may be lipidated by standard peptide synthesis to produce a full range of response without any extraneous adjuvant (ref. 8, ref. 9).

As mentioned above, MAPs have a number of other applications. Important areas are immunoassays and serodiagnosis of e.g. malaria, cirrhosis, HIV-1, schistoma mansoni, systemic lupus erythematosus, and Epstein-Barr virus (EBV).

Most studies of MAPs have been carried out using tetra- or octameric MAP. However, it is generally found that with peptides composed of more than 15 amino acids, no real advantage is obtained using an octameric as compared to a tetrameric MAP (ref. 2).

In general, two methods can be used for MAP synthesis, namely the direct route and the indirect route. In both cases, the C-terminal core is first assembled on a solid support using diprotected Boc-Lys(Boc) in the case of Boc chemistry, or Fmoc-Lys(Fmoc) in the case of Fmoc chemistry in order to obtain the desired degree of branching.

In the direct route, synthesis is continued by stepwise assembly
of the particular peptide on the lysinyl core by the well-known
Merrifield method. This stepwise method produces peptide antigens
with a C to N orientation. B and T cell epitopes may be synthesised
in tandem, or, alternatively, each epitope can be synthesised on
the different lysine arms of the core using orthogonal deprotection
methods. Such methods include i.a. Boc-Fmoc, and Fmoc-Aloc lysines.
Although this approach for MAP synthesis is convenient, the quality
of the final product may be questionable and problems of obtaining
a well defined product have been discussed (ref. 10). Problems
arise during chain assembly because MAPs are macromolecules where
each single chain may interact with each other, preventing high
efficiency couplings and leading to deletion sequences which
require effort to purify. Furthermore, the characterisation of
these products by, e.g. electrospray mass spectroscopy (ES-MS) is
also difficult and as a result, multi-dendritic peptide immunogens
have often been used without full characterisation. In the recent
investigations by Keah et al. (ref. 10), a capping procedure after
each amino acid coupling has been introduced in MAP synthesis,
which improves the yield of the desired multi-dendritic immunogens.
For analysis, an enzymatic (tryptic) digestion method with the MAP
construct, followed by characterisation of the enzymatic digest by
reversed phase high-performance liquid chromatography-ES-MS method
has been developed (ref. 10).

For many immunisations with MAP, the heterogeneity is not
necessarily critical but for clinical applications, it is
absolutely necessary to have chemically unambiguous MAPs without
side products.

These considerations led to the development of indirect methods for
MAP synthesis, in which methods purified unprotected peptide
segments are coupled to the core matrix. Two general methods for

this type of ligation have been developed which, respectively, are based on thiol and carbonyl chemistries.

The thiol chemistry may be carried out by incorporation of chloroacetyl group(s) on the lysine matrix and subsequent coupling to a purified, synthetic N-terminal cysteinyl peptide to yield a MAP with unambiguous structure (ref. 11, ref. 12). The reverse placement may be achieved with thiol on the core matrix by using an S-acetyl group on the lysinyl core and the haloacetyl group on the peptide (ref. 13). The use of disulphide bond formation for MAP synthesis is an alternative which is a well-known process in protein and peptide chemistry (ref. 2). A drawback in this type of chemistry is among others interference with cysteine present in the native antigen.

In the carbonyl chemistry, condensation takes place between a carbonyl group and a weak base. There are two types of weak bases which may be used for this reaction. The first includes hydroxylamines and hydrazines, and the second includes 1,2-disubstituted moieties such as 1,2-aminoethanthiol and 1,2-aminoethanol. The two last groups are found in N-terminal cysteine and threonine, respectively, and the condensation products are thiazolidine and oxazolidine. The carbonyl group on the core matrix may be obtained by periodate oxidation of N-terminal serine, threonine or cysteine.

Several investigations along these approaches for MAP construction have been carried out to develop other methods for preparing peptide dendrimers providing flexibility for N or C attachment to the matrix core as reviewed by Tam (ref. 2). The processes may need optimised conditions and a major concern by the use of thiols in the thiazolidine formation is a side reaction due to oxidation to disulphides, leading to impure products.

In the direct MAP synthesis route with stepwise assembly of the desired peptide on the lysinyl core a peptide antigen having C to N orientation is obtained. Such a MAP may be useful for ELISA applications, where antigen recognition by an antibody is directed at the N-terminal or preferably close to the N-terminal, while recognition of the C-terminal may be hindered. This is e.g. the case with the dominant epitope of the Outer surface protein C (OspC) from Borrelia burgdorferi sensu lato causing Lyme borreliosis. Epitope mapping of OspC using sera from patients with neuroborreliosis led to identification of one single major immunodominant epitope within the C-terminal ten amino acid residues, the decapeptide PVVAESPKKP. Peptide binding studies and alanine scanning revealed a critical role for the PKKP-sequence and in particular its terminal carboxyl group for the binding of IgM antibodies from patients with Lyme borreliosis. Thus substitution of the C-terminal proline or replacement of the carboxy group with a carboxamido group greatly reduced the ability of the peptides to compete with OspC19-207 for the binding of IgM antibodies in all five sera investigated. This significant fact clearly exclude a MAP formed by stepwise synthesis on a lysine core as this results in C-terminal attachment to the core matrix.

From the above discussion, it is clear that a system providing a choice between C-, and C- and N-terminal presentation of sequences, and at the same time being easily performed and yielding improved products suitable for various applications, is needed.

SUMMARY OF THE INVENTION

The present invention provides novel methods for preparing LPAs enabling presentation of desired sequence(s). In a further aspect, the present invention relates to a method for preparing LPAs enabling presentation of desired sequences(s) and chemical moie-

ties.

In another aspect, the present invention relates to an LPA obtainable by the method.

In yet a further aspect, the present invention relates to immunological compositions and vaccines, as well as a method of generating antibodies in an animal, including a human being.

Furthermore, the present invention relates to the use of such LPAs in various applications and kits.

The present invention is described in detail below.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the MALDI-TOF MS spectrum of the crude product $NH_2CH(CH_2CO\text{-}ProValValAlaGluSerProLysLysPro\text{-}OH)_2$ (also denoted $NH_2CH\text{-}(CH_2CO\text{-}Seq.\ ID\ 1\text{-}OH)_2$) [LPA-III] of Example 3.

Figure 2 shows the MALDI-TOF MS spectrum of the crude product $NH_2CH(CH_2CO\text{-}ProValValAlaGluSerProLysLysPro\text{-}OH)_2$ (also denoted $NH_2CH(CH_2CO\text{-}Seq.\ ID\ 1\text{-}OH)_2$) [LPA-III] of Example 3.

Figure 3 shows the HPLC chromatogram of crude H-ProValValAlaGluSer-ProLysLysPro-OH, cf. Example 3. (also denoted H-Seq. ID 1-OH)

Figure 4 shows the HPLC chromatogram of crude $NH_2CH(CH_2CO\text{-}ProValVal\text{-}AlaGluSerProLysLysPro\text{-}OH)_2$ (also denoted $NH_2CH(CH_2CO\text{-}Seq.\ ID\ 1\text{-}OH)_2$) [LPA-III] of Example 3.

Figure 5 shows the HPLC chromatogram of the HPLC purified product $NH_2CH(CH_2CO\text{-}ProValValAlaGluSerProLysLysPro\text{-}OH)_2$ also denoted

NH$_2$CH(CH$_2$CO-Seq. ID 1-OH)$_2$) [LPA-III] of Example 3.

Figure 6 shows the MALDI-TOF MS spectrum of the crude product H-Lys-NHCH(CH$_2$CO-ProValValAlaGluSerProLysLysPro-OH)$_2$ (also denoted H-Lys-NHCH(CH$_2$CO-Seq. ID 1-OH)$_2$) [LPA-IV] of Example 4.

Figure 7 shows the ELISA results from Example 13.

Figure 8 shows the ELISA results from Example 14.

Figure 9 shows the MALDI-TOF MS spectrum of the crude product CH$_2$(CH$_2$CO-β-Ala-β-AlaLysGluProAsnLysGlyValAsnProAspGluValβAla)$_2$ also denoted CH$_2$(CH$_2$CO-β-Ala-β-Ala-Seq. ID 4-β-Ala)$_2$ [LPA-VII] of Example 7.

Figure 10 shows the MALDI-TOF MS spectrum of the gel filtrated product HC(CH$_2$CO-LysGluProAsnLysGlyValAsnProAspGluValβAla)$_2$COOH also denoted HC(CH$_2$CO-Seq. ID 4-β-Ala)$_2$COOH [LPA-VIII] of Example 8.

Figure 11 shows the HPLC chromatogram of the gel filtrated product HC(CH$_2$CO-LysGluProAsnLysGlyValAsnProAspGluValβAla)$_2$COOH also denoted HC(CH$_2$CO-Seq. ID 4-βAla)$_2$COOH [LPA-VIII] of Example 8.

Figure 12 shows the MALDI-TOF MS spectrum of the crude product Fmoc-NHCH(CH$_2$CO-AspArgValTyrIleHisProPheHisLeu-NH$_2$)$_2$ also denoted Fmoc-NHCH(CH$_2$CO-Seq. ID 5-NH$_2$)$_2$ [LPA-IX] of Example 10.

Figure 13 shows the HPLC chromatogram of the crude product Fmoc-NHCH(CH$_2$CO-AspArgValTyrIleHisProPheHisLeu-NH$_2$)$_2$ also denoted Fmoc-NHCH(CH$_2$CO-Seq. ID 5-NH$_2$)$_2$ [LPA-IX] of Example 10.

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect, the present invention provides a novel method for preparing an LPA enabling presentation of desired sequence(s), which method comprises the steps of

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(a)   providing by solid phase synthesis or fragment coupling ligand(s) comprising the desired sequence(s), the ligand(s) being attached to a solid phase,

10   (b)   if necessary, deprotecting any N-terminal amino groups while the ligand(s) are still attached to the solid phase,

(c)   reacting the ligand(s) having unprotected N-terminal amino groups with an achiral di-, tri- or tetracarboxylic acid so
15   as to provide a construct having a ring structure, and

(d)   cleaving the construct from the solid phase so as to provide an LPA comprising ligand(s) having free C-terminal groups.

20   In a further aspect, the present invention provides a method for preparing an LPA enabling presentation of desired sequence(s), or desired sequence(s) and chemical moieties, which method comprises the steps of

25   (c^1)   if present, prior to step (d), deprotecting any N-protected amino groups originating from the carboxylic acid used in step (c),

(c^2)   continuing the solid phase synthesis or fragment coupling so
30   as to provide ligand(s) comprising desired sequence(s) having at least one N-protected N-terminal amino group and/or attaching chemical moieties, and

(c³)   deprotecting, if present, any N-terminal amino group(s)
prior to or after step (d).

A unique feature of the method according to the present invention
is the step providing a ring structure. Surprisingly, by the method
according to the present invention it is possible to prepare very
long ring systems interconnected by reaction with the achiral di-,
tri- or tetracarboxylic acid. The ring structure formed between
desired sequences further enables additional presentation of
desired sequence(s) and chemical moieties. Thus, the method of the
invention is easily performed and, as mentioned above, step (c¹)
and (c²) enable further presentation of desired sequences and/or
chemical moieties. The desired sequences of step (c²) may be
identical or different from the sequences of step (a). Thus, LPAs
are suitable and very flexible systems for polyfunctional
constructs, and furthermore, products of high purity are obtained.

Formation of double chain peptides having hemoregulatory activity
is disclosed in WO 93/24523 (ref. 14). The two peptide chains are
joined together at the Cα-atoms of non-terminal amino acids in
equivalent positions in each peptide chain via a carbon-carbon bond
or via divalent bridging group -A- where -A- is a C_{1-6} carbon chain.
The double chain peptides are constructed from a single chain of
the general formula R^a-R^b-R^c-R^d-$(R^e)_n$-R^f where R^a, R^b, R^c, and R^d
represent selected amino acids and R^e is -NH-CH(R)-CO-, R^f
represents lysine, arginine or glycine and n is 0 or 1. The
bridging group A is attached to the Cα-atoms of amino acids R^d and
bridging is accomplished by acylating the synthesis resin bearing
the desired N-deprotected C-terminal residue with one half
equivalent of Fmoc-protected diamino dicarboxylic acid of such a
type as 2,7-diaminosuberic acid or 2,9-diaminosebacic acid with the
aid of coupling agents DCC (dicyclohexyl carbodiimide) and HOBt.
After deprotection peptide synthesis is continued with the

remaining amino acids depending on the sequence in question. It is a characteristic and a necessary condition of the employed amino acids in order to obtain the claimed biologically active compounds that they contain two chiral centres. Furthermore, the peptide chains which are assembled with the said diamino dicarboxylic acid are limited to two amino acids each according to the depicted formula.

In recent investigations coupling with half equivalent of protected diamino dicarboxyl acids with synthesis resin bearing an N-deprotected C-terminal residue with up to three amino acids in the peptide chain has been reported (ref. 15, ref. 16). As to the conditions of the bridging technique it can be learned from the 1993 report by Alberts et al. (ref. 17) that bridging with half equivalent 2,7-bis(Boc-amino) suberic acid coupled to one equivalent of lysine attached to the synthesis resin (2Cl-Z) is slow and takes place over up to 4 days. Thus, ring formation of longer amino acid sequences may be similarly or more difficult requiring activation conditions which could prevent formation of well defined products with optically active bridging compounds.

The review by Tuchscherer and Mutter (ref. 18) deals with template-based de novo design of synthetic proteins (so-called TASP's, template-assembled synthetic proteins). De novo design of polypeptide sequences with a three-dimensional structure necessary for many biological functions is limited by the complex folding process of the polypeptide sequences. The problem of folding can be bypassed through constructing protein-like molecules with a "built in" device for intramolecular folding, namely proteins of non-natural chain architecture, i.e. TASP's. The concept is to use topological templates to introduce tertiary structures of the peptide sequences by directing attached peptide blocks to a predetermined three-dimensional packing arrangement. This method

provides synthetic proteins in which a predetermined backbone confirmation act as host for selective attachment of functional sites (e.g. amino acid side chains or peptides) in spatially defined positions via a spacer group. An early example of this method is the use of β-D-glucose as a template (scaffold) for the design of a non-peptidic mimic of the somatostatin agonist SRIF. Covalent attachment of the amino acid side chains of Phe, Trp and Lys to D-glucose derivatives resulted in a molecule recognised by G-protein coupled receptors. The concept and purpose described in this reference is clearly different from the concept of the present invention. There is no mentioning of synthesis or assembly of ligands using achiral di-, tri- or tetracarboxylic acids.

In WO 94/02506 (ref. 19) MAP systems are disclosed. The MAPs are constructed with the following structure: Peptide antigen-core-linker-lipid anchor-resin. The core is based on lysine technology with four lysine arms onto which the peptide is assembled by conventional stepwise synthesis by reaction of a terminal functionality, e.g. a carboxylic acid group or an amine group. Different active substituents may be included in the molecule, thus providing MAPs with multiple antigenicities. In the document, the use of di-, tri- or tetracarboxylic acids is not mentioned or suggested. Thus, the present invention is clearly based on a different concept.

In WO 98/32469 (ref. 20) compounds comprising a linear, branched or dendrimeric polymer backbone are disclosed. At least one reporter molecule is linked thereto. The polymer backbone comprises a plurality of amine-containing acids. The compounds are useful as therapeutic and diagnostic agents, in particular in medical imaging techniques. The concept is based on the recognition that co-polymers of amino acids carrying or attached to one or more reporter molecules (e.g. chelating moieties or absorbers) are

particularly suitable for diagnostic or therapeutic use. Dendrimeric polymers are the preferred backbone moieties. The compounds are formed from monomers which act as branching sites, and with each successive branching a new so-called generation is

5   formed. The dendrimeric backbone molecule preferably comprises a multiplicity of native or non-native amino acid residues arranged so as to extend radially outwards from a central core moiety. The amino acids may be terminally bonded directly or attached via a linking group to one or more reporter groups. Alternatively, these

10   may be terminally branched by the addition of further amino acid residues. The compounds are prepared using conventional stepwise solution synthesis. The use of di-, tri- or tetracarboxylic acids as in the present invention in the preparation is not disclosed or suggested, and, thus, the present invention is based on another

15   principle. The structure of the resulting compounds are clearly different from the LPAs of the present invention.

As mentioned previously, the publication by Tam (ref. 2) concerns design and immunological application of MAP's. The review focuses

20   on the recent progress achieved in MAP technology. I.a. cascade and pennant designs of core matrices for branched peptides are described including cascade and asymmetrical MAP with branching lysine, cascade and symmetrical MAP, pennant branched peptide, and polylysine as carrier. The review article does not disclose

25   synthesis using di-, tri- or tetracarboxylic acids.

The method of the present invention is based on assembly of two identical chains attached to a solid phase by means of achiral dicarboxylic, tricarboxylic or tetracarboxylic acids. By using

30   achiral compounds racemisation problems are avoided, thus allowing rigorous activation conditions for difficult reactions. With the exception of glycine all amino acids have a α-carbon atom including the amino dicarboxylic acids aspartic and glutamic acid. The use

of chiral acids leads to racemisation, i.e. in stead of obtaining
a single product, a mixture of diastereomers will be obtained, the
extent varying with reaction conditions. For clinical applications,
this is unacceptable and formation of clinically harmful side-

5   products is a potential risk. Racemisation problems arise upon
activation of the carboxylic group of a chiral coupling agent, and
in particular when the procedure is performed in the presence of
a base, during long reaction times and/or when the reaction is
carried out at elevated temperatures.

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Suitable achiral di-, tri- and tetracarboxylic acids to be used in
the present method have the general formula

$X[(A)_nCOOH][(B)_mCOOH]$

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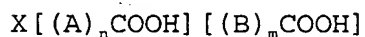
wherein n and m independently are an integer of from 1 to 20, X is
HN, A and B independently are a substituted or unsubstituted C_{1-10}
alkyl, a substituted or unsubstituted C_{2-10} alkenyl, a substituted
or unsubstituted cyclic moiety, a substituted or unsubstituted

20   heterocyclic moiety, or a substituted or unsubstituted aromatic
moiety, or A and B together form a substituted or unsubstituted
cyclic moiety, a substituted or unsubstituted heterocyclic moiety,
or a substituted or unsubstituted aromatic moiety, or

25   n and m are 0 or an integer of from 1 to 20, X is $H_2N(CR_2)_pCR$, or
$RHN(CR_2)_pCR$, wherein p is 0 or an integer of from 1 to 20, wherein
each R is H, a substituted or unsubstituted C_{1-10} alkyl, a
substituted or unsubstituted C_{2-10} alkenyl, a substituted or
unsubstituted cyclic moiety, a substituted or unsubstituted

30   heterocyclic moiety, or a substituted or unsubstituted aromatic
moiety, and A and B are both a substituted or unsubstituted C_{1-10}
alkyl, a substituted or unsubstituted C_{2-10} alkenyl, a substituted
or unsubstituted cyclic moiety, a substituted or unsubstituted

heterocyclic moiety, or A and B together form a substituted or unsubstituted cyclic moiety, a substituted or unsubstituted heterocyclic moiety, or a substituted or unsubstituted aromatic moiety, or

n and m are 0 or an integer of from 1 to 20, X is $HO(CR_2)_pCR$, $HS(CR_2)_pCR$, halogen-$(CR_2)_pCR$, $HOOC(CR_2)_pCR$, $ROOC(CR_2)_pCR$, $HCO(CR_2)_pCR$, $RCO(CR_2)_pCR$, or $[HOOC(A)_n][HOOC(B)_m]CR(CR_2)_pCR$, wherein p is 0 or an integer of from 1 to 20, each R independently is H, a substituted or unsubstituted C_{1-10} alkyl, a substituted or unsubstituted C_{2-10} alkenyl, a substituted or unsubstituted cyclic moiety, a substituted or unsubstituted heterocyclic moiety, or a substituted or unsubstituted aromatic moiety, and A and B are both a substituted or unsubstituted C_{1-10} alkyl, a substituted or unsubstituted C_{2-10} alkenyl, a substituted or unsubstituted cyclic moiety, a substituted or unsubstituted heterocyclic moiety, or A and B together form a substituted or unsubstituted cyclic moiety, a substituted or unsubstituted heterocyclic moiety, or a substituted or unsubstituted aromatic moiety, or

n and m are 0 or an integer of from 1 to 20, X is $H_2N(CR_2)_p$, $RHN(CR_2)_p$, $HO(CR_2)_p$, $HS(CR_2)_p$, halogen-$(CR_2)_p$, $HOOC(CR_2)_p$, $ROOC(CR_2)_p$, $HCO(CR_2)_p$, $RCO(CR_2)_p$ or $[HOOC(A)_n][HOOC(B)_m]$, wherein p is 0 or an integer of from 1 to 20, wherein each R independently is H, a substituted or unsubstituted C_{1-10} alkyl, a substituted or unsubstituted C_{2-10} alkenyl, a substituted or unsubstituted cyclic moiety, a substituted or unsubstituted heterocyclic moiety, or a substituted or unsubstituted aromatic moiety, and A and B together form a substituted or unsubstituted cyclic moiety, a substituted or unsubstituted heterocyclic moiety, or a substituted or unsubstituted aromatic moiety.

The term C_{1-10} alkyl is intended to comprise straight or branched

chain alkyl groups having 1-10 carbon atoms, e.g. methyl, ethyl, propyl, isopropyl, butyl, and tert-butyl.

The term C_{2-10} alkenyl is intended to comprise straight or branched chain alkenyl groups having 2-10 carbon atoms, e.g. ethenyl, propenyl, isopropenyl, butenyl, and tert-butenyl.

The term cyclic moiety is intended to comprise e.g. cyclohexan, and cyclopentane.

The term aromatic moiety is intended to comprise e.g. phenyl.

The expression A and B forms a cyclic, heterocyclic or aromatic moiety is intended to comprise e.g. cyclohexan, piperidine, benzene, and pyridine.

By reaction with a carboxylic acid, a construct of the type

$X(CO\text{-}sequence)_2\text{-}solid$ phase

is obtained, wherein X is as defined above.

By the term sequence, a peptide comprising naturally occurring and/or non-naturally occurring amino acids, a PNA-sequence, or a peptidomimetic is meant. Naturally occurring amino acids are L- and D-forms of the 20 amino acids found in nature. Non-naturally occurring amino acids are e.g. modified naturally occurring amino acids. The term sequence is further intended to comprise one or more of such sequences. Examples of suitable peptidomimetics are given by Marshall in ref. 21 and include oligo(N-substituted glycines), oligo carbamates, oligosulfones, and oligosulfoxides. Peptidomimetics also include peptoides and peptomers.

The term chemical moieties is intended to comprise an entity enhancing the solubility or immunogenecity of the LPA, an entity for directing the LPA to its target or a marker.

5   The group X permits directly or indirectly continued stepwise synthesis or fragment coupling of the same sequence, or of one or more different sequences and/or chemical moieties. In the case of peptides, LPAs with N to C orientation, or with simultaneous N to C and C to N presentation of the sequences are obtained. This is

10   a unique feature of the method of the present invention. Continued synthesis or fragment coupling (fragment attachment) of a sequence different from the first such as a B or T cell epitope affords a chimeric product.

15   In the case where X comprises a temporarily protected amino function, synthesis or coupling can be carried out directly after deprotection. Suitable activation of all carboxy-containing groups providing effective formation of the ring system can be ensured using half equivalent carboxylic acid. In case of tri- or

20   tetracarboxylic acids, the activated carboxy group may further be derivatised with a diamine such as ethylenediamine or an amine suitably functionalised for further reactions such as a mercapto-, an oxy-, an oxo or carboxyl group. In the case of a diamine, peptide synthesis or fragment coupling can be continued directly

25   according to the desired sequence or chemical moiety. In a preferred embodiment, the Fmoc-protection strategy is used, but any amino protection group may be used depending on the synthesis or coupling strategy. Examples are the Boc-protection group strategy.

30   Since the continued stepwise synthesis or fragment coupling is performed with one or in case of a bifunctional chemical moiety such as lysine with two amino groups, it has surprisingly been found that a much better result can be obtained as compared to

conventional tetrameric or octameric MAP. This is believed to be due to less interchain interaction. Furthermore, optimal peptide synthesis procedures or coupling procedures can be used for the single chains attached to the solid phase, and their homogeneity can be verified prior to forming the LPA. Cleavage from the solid phase and simultaneous side-chain deprotection can be performed by standard peptide synthesis procedures. A final LPA product may thus be obtained having optimal and well defined composition with possible by-products of less than half the molecular weight of the target molecule. Purification by standard chromatography methods such as HPLC or gel filtration can easily be performed, if desired or needed.

Favourable di-, tri- and tetracarboxylic acids for providing the ring structure are e.g. imino diacetic acid, 2-amino malonic acid, 3-amino glutaric acid, 3-methylamino glutaric acid, 3-chloro glutaric acid, 3-carboxymethyl glutaric acid, 3-methoxy-carbonyl glutaric acid, 3-acetyl glutaric acid, glutaric acid, tricarballylic acid, 3,4-bis-carboxymethyl adipic acid, 4-(2-carboxyethyl)-pimelic acid, (3,5-bis-carboxymethylphenyl)-acetic acid, 3,4-bis-carboxymethyl-adipic acid, benzene-1,2,4,5-tetra carboxylic acid, 4-(3-carboxy-allylamino)-but-2-enoic acid, 4,4'-imino-dibenzoic acid, 1,4-dihydropyridine-3,5-dicarboxylic acid, 5-amino isophthalic acid, 2-chloro malonic acid, 3-hydroxy glutaric acid, and benzene-1,3,5-tricarboxylic acid.

In the following, the individual steps of the method according to the present invention are described in further detail. Furthermore, apart from the general procedure, specific embodiments are given.

Step (a)

Solid phase synthesis has been known for a long time and can be

carried out according to well-known standard procedures. Solid phase synthesis of peptides is e.g. described in WO 98/11125 (ref. 22). Solid phase synthesis of PNA sequences is e.g. described in WO 98/15648 (ref. 23).

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Solid phase synthesis is generally carried out as described in the literature. However, a brief summary is given below.

The solid phase is preferably selected from functionalised resins

10 such as functionalised polystyrene, polyacrylamide, polyethyleneglycol, cellulose, polyethylene, latex or dynabeads.

If desired, C-terminal amino acids may be attached to the solid support by means of a linker such as 2-Cl-trityl, 2,4-dimethoxy-4'-

15 hydroxybenzophenone, 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB), 4-hydroxymethylbenzoic acid, 4-hydroxymethylphenoxyacetic acid (HMPA), 3-(4-hydroxymethylphenoxy)propionic acid or p-[(R,S)-α-[1-(9H-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid (AM).

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The synthesis may be carried out batch-wise or continuously on an automated or semi-automated peptide synthesiser.

The individual coupling steps may be performed in the presence of

25 a solvent, e.g. selected from acetonitrile, N,N-dimethylformamide (DMF), N-methylpyrrolidinone (NMP), dichloromethane (DCM), trifluoroethanol (TFE), ethanol, methanol, water, mixtures of the mentioned solvents with or without additives such as perchlorate or ethylenecarbonate.

30

The individual couplings between two amino acids, an amino acid and the earlier formed sequence or fragment may be carried out according to usual condensation methods such as the azide method,

mixed acid anhydride method, symmetrical anhydride method, carbodiimide method, or active ester method using pentafluorophenyl (Pfp), 3,4-dihydro-4-oxobenzotriazin-3-yl (Dhbt), benzotriazol-1-yl (Bt), 7-azabenzotriazol-1-yl (At), 4-nitrophenyl, N-hydroxysuccinic acid imido esters (NHS), acid chlorides, acid fluorides, in situ activation by O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), benzotriazolyl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP), benzotriazolyl-N-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), and 7-azabenzotriazolyl-N-oxytripyrrolidinophosphonium hexafluoro-phosphate (PyAOP).

Examples of temporary protection groups are Fmoc and Boc in the case of Cα-amino protection. Examples of side-chain protection groups are Boc, tBu, Z, and Cbz. Examples of orthogonal protection groups are Fmoc, Boc, Mtt, Dde, All, ODmab, and Aloc.

Fmoc groups may be removed by means of an amine such as piperidine or diazabicyclo[5,4,0]undec-7-ene (DBU).

Side-chain protective groups may be removed by means of an acid such as TFA, TFMSA, HBr, HCl or HF.

To facilitate the reaction, the reaction mixture may heated to a temperature of about 60°C.

Fragment coupling (fragment coupling or fragment condensation) may be performed according to standard procedures, e.g. as described in ref. 24. Accordingly, fragments may be synthesised on a solid phase, cleaved from the solid phase with full preservation of

protecting groups, purified and characterised. Synthesis of the fragments may be carried out as described above. Suitable fragments may also be obtained by other techniques including enzymatic and recombinant techniques, and coupled as described above.

Step (b)

During solid phase synthesis or fragment coupling, N-terminal amino groups within the amino acid building blocks or side-chain amino groups of PNA moieties or peptidomimetics may be protected by suitable protecting groups. Examples of temporary protection groups are Fmoc and Boc in the case of $C\alpha$-amino protection. Examples of orthogonal protection groups are Mtt, Dde, All, ODmab, and Aloc.

If any protected amino groups are present, such may suitably be deprotected, while the sequences are still attached on the solid phase. Suitable deprotecting agents are those usually used in the peptide chemistry and include piperidine, DBU or TFA in the case of temporary protection groups, and hydrazine, Pd/AcOH/NMM, or TFA in the case of orthogonal protection groups.

Step (c)

A ring structure is provided using a di-, tri- or tetracarboxylic acid. Formation of the ring structure is carried out under conditions enabling cyclisation of N-terminal amino groups of the desired sequences.

Suitable achiral di-, tri- and tetracarboxylic acids are given above.

Suitably, the carboxylic acid may be used in an amount of approximately 0.4 to 0.6, preferably 0.5, equivalents carboxylic

acid/peptide-resin.

Cyclisation may alternatively be carried out using 1:1 orthogonal N-terminal protection. After deprotection of 0.5 eq of the peptide-chain amino-groups the di-, tri- or tetracarboxylic acid in excess is activated and coupled. After orthogonal deprotection cyclisation is achieved by activation of the second carboxylic acid group. Examples of orthogonal protection groups are Fmoc, Boc, Mtt, Dde, All, ODmab, and Aloc.

Activation may be carried out according to usual condensation methods such as the azide method, mixed acid anhydride method, symmetrical anhydride method, carbodiimide method, or active ester method using Pfp, Dhbt, Bt, At, 4-nitrophenyl, NHS esters, acid chlorides, acid fluorides, in situ activation by HATU, HBTU, TBTU, BOP, PyBOP, and PyAOP.

As already mentioned, the composition of the ligand may be verified and a sequence of the best possible purity may be achieved before the cyclisation procedure. If a test of the so obtained product is found to contain monomeric substitution product, the procedure can be repeated to achieve a possible better yield. Alternatively, in the case where free peptide chains are present after cyclisation they may be terminated by e.g. acetylation.

The reaction mixture may be heated up to about 60°C in order to facilitate the reaction. These harsh reaction conditions can be applied since an achiral carboxylic acid is used. If a chiral carboxylic acid were used, elevated reaction temperatures would promote racemisation.

Step (d)

The resulting product, the construct, i.e. the LPA, is cleaved from the solid phase, whereby an LPA comprising ligands having free C-terminal groups are provided. Deprotection of any side-chain protecting groups can, if desired, be carried out simultaneously with cleavage from the solid phase.

Cleavage from the solid phase can be carried out using well-known agents for this purpose such as TFA, TFMSA, HBr, HCl, HF, ammonia, hydrazine, an alkoxide or a hydroxide. Alternatively, the sequence is cleaved from the solid phase by means of photolysis.

Furthermore, because the method of the invention provides an LPA of approximately the double molecular weight, standard chromatography methods such as HPLC or gel filtration can be used to obtain a single target LPA.

Step (c^1)

If a N-protected amino group originating from the carboxylic acid used in step (c) is present, such may be deprotected. Deprotection may be carried out using well-known conditions and agents. The amino group may suitably be protected by Fmoc or Boc. Suitable deprotecting agents are those usually used in the peptide chemistry and include piperidine, and DBU or TFA.

Step (c^2)

Additional presentation of ligands comprising desired sequence(s) and/or chemical moieties may be provided by synthesis or coupling as described above.

Dicarboxylic acids
By the deprotection in step (c^1), a free amino group is obtained.

The side chains of the sequence amino acid groups are preferably still fully protected.

Synthesis or fragment coupling may then be continued on the free amino group to provide a ligand comprising one or more desired sequences, said ligand having N-protected N-terminal amino group(s). Synthesis or coupling can be carried out using peptide chemistry, synthetic procedures appropriate for PNA, or procedures appropriate for peptidomimetics. Alternatively, synthesis or coupling may be carried out so as to incorporate a chemical moiety. Such chemical moieties may suitably be a fatty acid or another compound capable of improving the solubility of the LPA or immunogenicity (adjuvant effect), an entity suitable for directing the LPA to its target, a marker, or any other group providing specific desirable properties. Lysine and amino hexanoic acid are examples of such other groups and may be used for chain extension.

Synthesis or fragment coupling may also be continued on the free amino group to provide a construct terminating with a diamino acid such as lysine, the α- and ε-amino groups of this diamino acid may be suitably protected (the protection groups being identical or different). Suitable protection groups (orthogonal protection groups) are Fmoc, Boc, Mtt, Dde, All, ODmab, and Aloc. Subsequently, solid phase synthesis or fragment coupling may be carried out on each of the amino groups (i.e. both the α- and the ε-amino group), or only on one of the amino groups (i.e. the α- or the ε-amino group) to provide a final bifunctional or trifunctional LPA. Solid phase synthesis may be carried out to provide sequences at both the α- and the ε-amino group, or only on the α- or the ε-amino group, and at the same time incorporating a chemical moiety on the other. Alternatively, chemical moieties may be incorporated on both the α- and the ε-amino group.

Examples of markers as chemical moieties are fluorophores, biotin, dinitro benzoic acid, haptens such as digoxigenin, enzymes such as HRP, alkaline phosphatases, and soya bean peroxidase, europium, and dyes such as rhodamines, cyanine dyes, coumarin, R-phycoerythrin (RPE) and allophycoerythrin.

Tri- and tetracarboxylic acids

Prior to step (d) described above, the free carboxylic group may be activated and functionalised by use of a suitable compound containing amino functions and/or thiol and/or hydroxy functions such as 1,2-diamino ethane, 2-aminoethanthiol, and 2-aminoethanol.

In case of functionalising using a compound having a diamino function, synthesis or coupling may then be continued on the free amino group so as to provide a desired sequence having a N-protected N-terminal amino group. Synthesis can be carried out using peptide chemistry or synthesis appropriate for PNA or procedures appropriate for peptidomimetics.

If the activated and functionalised construct terminates in an amino acid such as lysine, the α- and ϵ-amino groups may be suitably protected (the protection groups being identical or different). Suitable protection groups (orthogonal protection groups) are Fmoc, Boc, Mtt, Dde, All, ODmab, and Aloc. Subsequently, synthesis or coupling may be carried out on each of the amino groups (i.e. both the α- and the ϵ-amino group), or only on one of the amino groups (i.e. the α- or the ϵ-amino group) so as to provide a final bifunctional or trifunctional LPA. Solid phase synthesis or fragment coupling may be carried out to provide sequences at both the α- and the ϵ-amino group, or only on the α- or the ϵ-amino group and at the same time incorporating a chemical moiety on the other. Alternatively, chemical moieties may be incorporated at both the α- and the ϵ-amino group. Examples of

suitable chemical moieties are given above.

Step (c³)

5   Deprotection of any protected amino groups may be carried out prior
    to, subsequently to or simultaneously with the cleavage in step
    (d). Suitable cleavage agents are mentioned above under step (d).

    As mentioned above, a remarkable and advantageous feature by the
10 method provides an LPA with very well-defined composition as
    optimised synthetic or coupling procedures can be used for
    preparation of the sequences.

    The significant aspect of the use of amino or imino dicarboxylic
15 acids in step (c) is the readily possibility of continued synthesis
    or coupling. After removal of the temporary protecting Fmoc-group
    in solid phase-attached and side-chain protected product, coupling
    with a desired activated and protected amino acid component may be
    effected. A typical example is attachment of Fmoc-protected amino
20 hexanoic acid followed by standard Fmoc-deprotection with
    piperidine and coupling with biotin with common activating agents.
    The resulting product is useful for e.g. ELISA applications with
    avidin or streptavidin as coating agents.

25 The method provides i.a. LPAs presenting desired sequences with N
    to C orientation (using conventional terminology) (step (c)), and
    also simultaneously sequences with C to N orientation (in the case
    of incorporation of ligands comprising amino acid sequences) (step
    (c²)).

30

    In a preferred embodiment of the present invention, the ligands
    comprising the desired sequence(s) comprise all or part of one or
    more B-cell epitopes, all or part of one or more T-cell epitopes,

or all or part of one or more B- and T-cell epitopes (chimeras), or mimics thereof. The three-dimensional structure of a protein is often such that amino acids, which are located distant from each other in the one-dimensional structure, are located near to each other in the assembled protein. Within the meaning of the present context, the expression epitope is intended to comprise the one- and three-dimensional structure as well as mimics thereof. The term is further intended to include discontinuous B-cell epitopes.

In accordance with the present invention, the desired sequences may be derived from viruses, bacteria, toxins, allergens, autoimmune system-related agents, cancer-related agents, cell adhesion molecules, neurotropic factors, fungi or parasites.

The peptide ligands may have substitutions, deletions or additions as long as the intended function of the ligand is sufficiently preserved. By the term substitution is meant substitution of one or more amino acids with another amino acids. By the term deletion is meant removal of one or more amino acids from the sequence. By the term addition is meant insertion of one or more amino acids in the sequence. The sequence should have at least 40% identity, at least 50% identity, preferably at least 75% identity, and more preferably at least 95% identity, with the sequence from which it is derived.

In accordance with the present invention, the desired sequence may preferably be derived from

viruses such as Myxoviruses e.g. influenza virus, Paramyxoviruses e.g. parainfluenza virus, mumps, measles, and Newcastle disease, Picornaviruses e.g. poliovirus, coxsackievirus, echovirus and rhinovirus, Reoviruses, Poxviruses e.g. as small pox virus, Vaccinia virus, and cowpox virus, Papovaviruses e.g. polyoma virus,

papilloma virus and SV-40, Adenoviruses, Astroviruses, SRSV (small round-structures virus), Epstein-Barr virus (EBV), Parvoviruses e.g. Human Parvovirus B19, Herpesviruses e.g. herpes simplex virus, cytomegalovirus (CMV), Varicella virus (herpes zoster virus) and

5   Pseudorabies virus, Syncytial viruses e.g. human syncytial virus (HSV), Arboviruses e.g. yellow fever, and dengue fever, Leukoviruses e.g. Rous sarcoma virus, retroviruses e.g. HIV, and Hepatitis viruses e.g. Hepatitis A, Hepatitis B and Hepatitis C,

10   bacteria such as Mycobacterium spp. e.g. M. tuberculosis, M. bovis, M. africanum, M. microti, M. avium, M. intracellulare, M. kansasii, M. gordonae, M. paratuberculosis, and M. lepramurium, Borrelia spp. such as B. burgdorferi, in particular B. burgdorferi sensu stricto, B. garinii, B. afzelii, B. duttoni and B. recurrentis, Salmonella

15   spp. e.g. S. typhimurium, and S. typhi, Treponema spp. e.g. T. pallidum, Leptospira spp., Campylobacter spp. e.g. C. jejuni, Helicobacter spp. e.g. H. pylori, Pseudomonas spp., Legionella spp., Neisseria spp. e.g. N. gonorrhoea and N. menigitidis, Chlamydia spp. e.g. C. trachomatis, C. pneumonia and C. psittae,

20   Brucella spp., Enterobacter spp., Klebsiella sppl., Yersinia spp., Vibrio spp. e.g. Vibrio chloerae, Gardnerella spp., Rickettsia spp., Clostridium spp. e.g. C. difficile and C. botulinum, Lactobacillus spp., Listeria spp., and Mycoplasma spp. e.g. M. pneumoniae and M. hominis,

25

toxins such as C. dificile toxin A and B, and C. tatani tetanospasmin,

allergens such as pollen allergens originating from the taxonomic

30   order of Fagales, Oleales, Pinales, Poales, Asterales, or Urticales, a mite allergen originating from Dermatophagoides, a cockroach allergen, and animal allergen originating from cat, dog or horse, a venom allergen originating from the taxonomic order of

Hymenoptera, or a fungi allergens originating from the genera Alternaria and Cladosporium,

auto-immune system-related compounds such as TNFα,

cancer-related compounds,

cell adhesion molecules,

neurotropic factors,

fungi,

parasites such Plasmodium e.g. P. falciparum, P. malariae, P. vivax, and P. ovale, Leishmania e.g. Leishmania donovani, L. brasiliensis, L. mexicana and L. peruviana, or

a sequence homologous thereto.

Two bacterial species may be of particular interest, namely Borrelia spp. and Mycobacterium spp.

The tickborne spirochete Borrelia burgdorferi is the etiological agent of Lyme borreliosis, which is at present the most common vector-borne human disease in Europe and North America.

It is desirable to have access to an assay with a high diagnostic sensitivity already in the first stage of Lyme borreliosis, in order to diagnose and treat patients before they develop severe symptoms of the later stages of Lyme borreliosis.

According to Western blot studies there are only two B. burgdorferi antigens that meet the essential criterion of eliciting an early

and strong antibody response in the majority of patients. These are the B. burgdorferi flagellum and the outer surface protein C (OspC). Whereas the performance of EIAs (enzyme immuno assays) using purified native B. burgdorferi flagellum is well documented,

5 the reported experience with OspC EIAs is still limited.

WO 97/42221 (ref. 25) discloses that the 4 terminal amino acids Pro-Lys-Lys-Pro (Seq. 10 7) are essential in the immune reactivity between sera from patients suffering from early borreliosis and various OspC

10 derivatives. In order to obtain an effective diagnostic agent a polypeptide fragment which contains carboxyterminally a peptide with the general formula $A^5-A^4-A^3-A^2-A^1$, where A^1 and A^4, independently from each other, designate a residue of an amino acid, wherein a nitrogen atom capable of forming part of a peptide

15 bond is part of a ring structure; A^2 and A^3, independently from each other, designate residues or a positively charged or polar amino acid; and A^5 designates residues of any amino acid, such that the peptide of the formula above has a degree of sequence identity of at least 60% with the amino acid residue subsequence of Ser-Pro-

20 Lys-Lys-Pro (Seq. 10 8) is preferred. Such polypeptide fragments may advantageously be used in preparing a multimeric structure according to the invention. Particular preferred OspC carboxyterminal peptides are Val-Ala-Glu-Ser-Pro-Lys-Lys-Pro, Val- (Seq. 10 9) Val-Ala-Glu-Ser-Pro-Lys-Lys-Pro, and Pro-Val-Val-Ala-Glu-Ser-Pro-

25 Lys-Lys-Pro. (Seq. 10 10)
(Seq. 10 1)

In one embodiment of the present method, at least one of the sequences is derived from a sequence, wherein the C-terminal amino acids are important for an immune response.

30

In another embodiment of the present method, the desired sequence is derived from the OspC protein of Borrelia burgdorferi, or is a homologous sequence capable of reacting with anti-OspC antibodies,

or is capable of provoking an immune response. In particular, the LPA obtained provides a C-terminal presentation of the C-terminal sequence Pro-Lys-Lys-Pro of OspC.

(Seq, 16 7).

5   In accordance with the invention, the presentation of a carboxyterminal OspC related peptide as described above can be combined with presentation of another sequence relevant for diagnosing Lyme borreliosis. In particular, for early diagnosis of Lyme borreliosis sequences comprising relevant epitopes from the

10   flagellum are relevant. Other relevant sequences might be derived from OspA, OspB, OspD, OspE, OspF, Erp proteins, BmpA (P39), P100, P35, P37 and DbpA. In particular, sequences derived from OspC, the flagellum, BmpA, (P39) or P100 are suitable sequences for diagnostic uses. Sequences derived from OspA, OspC or DbpA are

15   suitable vaccine candidates.

In yet another embodiment, the desired sequence is derived from the flagellum of Borrelia burgdorferi or is a sequence homologous thereto capable of reacting with anti-flagellum antibodies

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In yet another embodiment of the present invention, the LPA providing C-terminal presentation of sequences derived from OspC of Borrelia burgdorferi further comprises desired sequence(s) derived from the flagellum of Borrelia burgdorferi.

25

In one embodiment of the present method, the desired sequence is derived from Mycobacterium tuberculosis. In a further embodiment, the LPA of the invention provides C-terminal presentation of the desired sequences of OspC derived from Borrelia burgdorferi, and

30   N-terminal presentation of desired sequence(s) derived from Mycobacterium tuberculosis. In particular, the desired sequence derived from M. tuberculosis may comprise the sequence of amino acids number 51 to 70 of ESAT-6 (ESAT-6, 51-70 sequence protein)

or the ESAT-6, 1-17 sequence protein of Mycobacterium tuberculosis (cf. WO 95/01441 ref. 26).

The present invention also concerns an LPA obtainable by the method defined above. The LPAs of the invention enables presentation of desired sequence(s), or presentation of desired sequence(s) and chemical moieties. Such sequences are suitably selected from a peptide comprising naturally occurring and/or non-naturally occurring amino acids, a PNA sequence, or a peptidomimetic. The chemical moieties are suitably an entity enhancing the solubility or immunogenicity of the LPA, or is an entity suitable for directing the LPA to its target, or a marker. In particular, the chemical moiety may be selected from fatty acids, antibodies or peptides for directing the LPA to its target, biotin, enzymes such as horse radish peroxidase, alkaline phosphatase and soya bean peroxidase, or nucleic acid sequences.

In one embodiment, the LPA of the invention comprises all or part of one or more B cell epitopes, all or part of one or more T cell epitopes, or all or part of one or more B and T cell epitopes, or a mimic thereof. In particular, at least one of the sequences may be derived from a sequence, wherein the C-terminal amino acids are important for an immunogenic response.

In another embodiment, the LPAs of the invention comprises sequences which are derived from viruses, bacteria, toxins, allergens, autoimmune system-related compounds, cancer related compounds, fungi or parasites, or a sequence homologous thereto.

In another embodiment, the LPAs of the invention are such, wherein the desired sequence is derived from the OspC protein of Borrelia burgdorferi. In particular, the LPA obtained may provide a C-terminal presentation of the C-terminal sequence Pro-Lys-Lys-Pro

(Seq ID 1) 2

of OspC.

Examples of suitable LPAs are

5   [LPA-I]:   FmocN(CH$_2$CO-ProValValAlaGluSerProLysLysPro-OH)$_2$,   also denoted FmocN(CH$_2$CO-Seq. ID 1-OH)$_2$,

    [LPA-II]: biotin-NH(CH$_2$)$_5$CON(CH$_2$CO-ProValValAlaGluSerProLysLysPro-OH)$_2$ also denoted biotin-NH(CH$_2$)$_5$CON(CH$_2$CO-Seq. ID 1-OH)$_2$,

10

    [LPA-III]:   NH$_2$CH(CH$_2$CO-ProValValAlaGluSerProLysLysPro-OH)$_2$ also denoted NH$_2$CH(CH$_2$CO-Seq. ID 1-OH)$_2$, and

    [LPA-IV]: H-Lys-NHCH(CH$_2$CO-ProValValAlaGluSerProLysLysPro-OH)$_2$ also

15   denoted H-Lys-NHCH(CH$_2$CO-Seq. ID 1-OH)$_2$.

In another embodiment of the LPA of the present invention, the desired sequence is derived from Mycobacterium tuberculosis. In a further embodiment of the LPA, C-terminal presentation of the

20   desired sequences derived from OspC of Borrelia burgdorferi, and N-terminal presentation of desired sequence(s) derived from Mycobacterium tuberculosis are provided.

In particular, the LPA providing C-terminal presentation of the C-

(Seq ID 7)

25   terminal Pro-Lys-Lys-Pro of OspC of Borrelia burgdorferi, and N-terminal presentation of ESAT-6 sequences from M. tuberculosis may be selected from

    [LPA-V]:   (HO-ProLysLysProSerGluAlaValValPro-COCH$_2$)$_2$CH-NH-Lys-

30   (GlnLeuAlaAsnAsnLeuGluThrAlaThrAlaAspTrpLysGlnGlnValGlyGlnTyr-H)$_2$, also denoted (HO-Seq. ID 1-COCH$_2$)$_2$CH-NH-Lys(Seq. ID 2-H)$_2$, and

    [LPA-VI]:   (HO-ProLysLysProSerGluAlaValValPro-COCH$_2$)$_2$N-Lys(AlaSer-

AlaAlaAlaGluIleGlyAlaPheAsnTrpGlnGlnGluThrMet-H)₂ also denoted (HO-Seq. ID 1-COCH₂)₂N-Lys(Seq. ID 3-H)₂.

Other examples of LPAs of the present invention are

[LPA-VII]:   CH₂(CH₂CO-β-Ala-β-AlaLysGluProAsnLysGlyValAsnPro-AspGluValβAla)₂ also denoted CH₂(CH₂CO-β-Ala-β-Ala-Seq. ID 4-β-Ala)₂ of Chlamydia trachomatis DnaK 357-368,

[LPA-VIII]: HC(CH₂CO-LysGluProAsnLysGlyValAsnProAspGluValβAla)₂COOH also denoted HC(CH₂CO-Seq. ID 4-βAla)₂COOH of Chlamydia trachomatis DnaK 357-368,

[LPA-IX]: Fmoc-NHCH(CH₂CO-AspArgValTyrIleHisProPheHisLeu-NH₂)₂ also denoted Fmoc-NHCH-(CH₂CO-Seq. ID 5-NH₂)₂ of Angiotensin-I,

[LPA-X]: Aloc-NHCH(CH₂CO-AspArgValTyrIleHisProPheHisLeu-NH₂)₂ also denoted Aloc-NHCH-(CH₂CO-Seq. ID 5-NH₂)₂ of Angiotensin-I, and

[LPA-XI]:   Fmoc-AspProThrGlnAsnIleProProGly-NHCH(CH₂CO-AspArg-ValTyrIleHisProPheHisLeu-NH₂)₂ also denoted Fmoc-Seq. ID 6-NHCH(CH₂CO-Seq. ID 5-NH₂)₂ (Angiotensin I extended with Chlostridium thermosaccharolyticum peptide sequence 19-27).

In yet a further aspect, the present invention relates to an immunological composition for raising an immune response in an animal, including a human being comprising an LPA as defined above. In one embodiment, the immunological composition may comprise an LPA as defined above i.a. in combination with an adjuvant.

The immunological composition may suitably be in the form of a vaccine. Such a vaccine may suitably be for oral or parenteral, e.g. subcutaneous, intramuscular, intradermal, nasal, or pulmonary

administration.

In another aspect, the present invention relates to a method for generating antibodies in an animal, including a human being, which method comprises administering to said mammal an antibody-generating amount of an LPA or an immunological composition as defined above.

Furthermore, the present invention relates to the use of an LPA as defined above for preparing a pharmaceutical composition for the treatment, alleviation or prophylaxis of diseases caused by viruses, bacteria, toxins, allergens, autoimmune system-related compounds, cancer related compounds, cell adhesion molecules, neurotropic factors, fungi or parasites.

Formulation of vaccines

Although it is preferred to insert a whole B- or T-cell epitope, it may in some cases be advantageous to insert a sequence comprising both the epitope as well as flanking regions from the protein from which the epitope is derived. The amino acids of a given epitope may be located near each other when the sequence is in the three-dimensional structure, but distant when the sequence is denatured. The sequence may be composed of the amino acids actually forming the epitope in the three-dimensional structure omitting all or part of the other amino acids.

According to the invention the LPAs may be used in vaccines against infections with pathogenic agents as described above. Strategies in formulation development of vaccines based on the products obtained by the method of the present invention generally correspond to formulation strategies for any other protein-based drug product. Potential problems and the guidance required to

overcome these problems are dealt with in several textbooks. The use of an adjuvant, e.g., aluminium hydroxide, aluminium phosphate (Adju-Phos), calcium phosphate, muramyl dipeptide analogue, or some of the more recent developments in vaccine adjuvants such as

5   biodegradable microparticles and Iscom's is a formulation challenge familiar to a pharmaceutical scientist working in this area.

Usually vaccines require the use of adjuvants. According to the invention the modified human TNFa molecules can be formulated with such appropriate adjuvants, e.g. aluminium phosphate (Adju-Phos)

10   or other alternative adjuvants such as aluminium hydroxide, calcium phosphate, muramyl dipeptide analogs, Iscom's or other known adjuvants used in mammalian vaccines.

Preparation of the vaccines according to the invention which contain peptide sequences as active ingredients is generally well

15   understood in the art, as exemplified by US 4 608 251 (ref. 27), US 4 601 903 (ref. 28), US 4 599 231 (ref. 29), US 4 599 230 (ref. 30), US 4 596 792 (ref. 31), and US 4 578 770 (ref. 32), all incorporated herein by reference. Typically, such vaccines are pre-pared as injectables either as liquid solutions or suspensions;

20   solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for

25   example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccines.

30

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of

administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the

5 active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of

10 solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10-95% of active ingredient, preferably 25-70%.

The LPA according to the invention may be formulated into the

15 vaccine as neutral or salt forms. Pharmaceutically acceptable salts include acid addition salts (formed with the free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic oxalic, tartaric, mandelic, and the like. Salts formed

20 with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

25

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, e.g., the capacity of the

30 individual's immune system to mount an immune response. Suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination with a preferred range from about 0.1 μg to 1000 μg, such as in the range from about 1 μg to 300 μg, and

especially in the range from about 10 μg to 50 μg. Suitable regimens for initial administration and booster shots are also variable but are typified by an initial administration followed by subsequent inoculations or other administrations.

5

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These are believed to include oral application on a solid physiologically acceptable base or in a physiologically

10 acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the age of the person to be vaccinated and, to a lesser degree, the size of the person to be vaccinated.

15 Some of the LPAs according to the present invention may be sufficiently immunogenic in a vaccine, but for some of the others the immune response will be enhanced if the vaccine further comprises an adjuvant substance.

20 Various methods of achieving adjuvant effect for the vaccine include use of agents such as aluminium hydroxide or aluminium phosphate (alum, Adju-Phos), commonly used as 0.05 to 0.1 percent solution in phosphate buffered saline, admixture with synthetic polymers of sugars (Carbopol) used as 0.25 percent solution,

25 aggregation of the protein in the vaccine by heat treatment with temperatures ranging between 70°C to 101°C for 30 second to 2 minute periods respectively. Aggregation by reactivating with pepsin treated (Fab) antibodies to albumin, mixture with bacterial cells such as C. parvum or endotoxins or lipopolysaccharide

30 components of gram-negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A) or emulsion with 20 percent solution of a perfluorocarbon (Fluosol-DA) used as a block substitute may also be employed. According to the

invention DDA (dimethyldioctadecylammonium bromide) is an
interesting candidate for an adjuvant, but also QuilA and RIBI are
interesting possibilities. Further possibilities are monophosphoryl
lipid A (MPL), and muramyl dipeptide (MDP). Other suitable
5   adjuvants are calcium phosphate, and muramyl dipeptide analogue.
Some of the more recent developments in vaccine adjuvants such as
biodegradable microparticles and Iscom's may also suitably be used.

Another highly interesting possibility of achieving adjuvant effect
10   is to employ the technique described in Gosselin et al., 1992
(which is hereby incorporated by reference). In brief, the
presentation of a relevant antigen can be enhanced by conjugating
such antigen to antibodies (or antigen binding antibody fragments)
against the Fcγ receptors on monocytes/macrophages. Especially
15   conjugates between antigen and anti-FcγRI have been demonstrated
to enhance immunogenicity for the purposes of vaccination.

Other possibilities involve the use of immune modulating substances
such as lymphokines (e.g. IFN-γ, IL-2 and IL-12) or synthetic IFN-γ
20   inducers such as poly I:C in combination with the above-mentioned
adjuvants.

In many instances, it will be necessary to have multiple
administrations of the vaccine, usually not exceeding six
25   vaccinations, more usually not exceeding four vaccinations and
preferably one or more, usually at least about three vaccinations.
The vaccinations will normally be at from two to twelve week
intervals, more usually from three to five week intervals. Periodic
boosters at intervals of 1-5 years, usually three years, will be
30   desirable to maintain the desired levels of protective immunity.

One reason for admixing the LPAs of the invention with an adjuvant
is to effectively activate a cellular immune response.

Diagnosis

When the LPAs are used for diagnosis, they may be applied in various immunoassays. Samples to be tested may be obtained from e.g. body fluids such as blood, serum, cerebro spinal fluid (CSF), pleural, pericardial, synovial, pus and bone marrow, sputum, laryngeal swabs, gastric lavage, bronchial washings, biopsies, aspirates, expectorates, urine, and tissue samples and further food samples, soil, and water samples as well as cultures thereof.

Apart from the use in vaccines, the LPA of the invention may be used as a diagnostic tool. Other biochemical uses of the LPA include epitope mapping, inhibitors, artificial protein, intracellular delivery, and affinity purification of antibodies.

When the LPA is used as a diagnostic tool, a sample is brought into contact with the LPA under suitable conditions, and any reaction between the LPA and the target material is observed or measured. Suitable assays for diagnostic purposes include immunosorbent assays such as ELISA, dot blot, flow cytometry and skin tests. Such assay formats may be carried out by procedures well-known to the person skilled in the art.

The present invention further concerns a kit for use in the diagnosis of infections caused by viruses, bacteria, toxins, allergens, auto-immune system-related compounds, cancer related compounds, fungi or parasites, which kit comprises an LPA as defined above together with means for detecting or visualising binding between the LPA and the substance to be detected. In one embodiment, the kit may be for use in diagnosing diseases caused by Borrelia burgdorferi sensu lato, and comprising an LPA as defined above together with means for detecting or visualising binding between the LPA and the substance to be detected.

The present invention is further illustrated by the following non-limiting examples.

EXAMPLES

5

General apparatus

HPLC conditions

HPLC was preformed on a Waters 600 E instrument equipped with a

10   Waters 996 Photodiode array detector with a Waters Radial Pak 8 x
100 mm C18 reversed-phase column. Buffer A was 0.1vol % TFA in
water and buffer B 90 vol% acetonitrile, 9.9 vol% water and 0.1
vol% TFA. The Buffers were pumped through the column at a flow rate
of 1.5 ml/min using a linear gradient from 0% - 70% B (20 min),

15   linear gradient from 70 - 100% B (1 min), isocratic 100% B (5 min).

Mass spectroscopy

MALDI TOF spectra were obtained on a Fisons TofSpec E instrument.
As matrix were in all cases used α-cyano-4-hydroxy-cinnamic acid.

20   Molecular ions were recorded as M·H+ or M·Na+ and M·K+ adduct ions.

Amino acid analysis

Amino acid analysis was performed with a Waters PICOTAG system.

25   Amino acid sequencing

N-Terminal amino acid sequence determination was performed with
Applied Biosystems 494 Protein Sequencer, Procise instrument.

Peptide synthesis

30

General procedures

Apparatus and synthetic strategy

Peptides were synthesised batch-wise in a polyethylene vessel

equipped with a polypropylene filter for filtration using 9-fluorenylmethyloxycarbonyl (Fmoc) or tert-butyloxycarbonyl, (Boc) as N-α-amino protecting group and suitable common protection groups for side-chain functionalities.

Solvents

Solvent DMF (N,N-dimethylformamide, Riedel de-Häen, Germany) was purified by passing through a column packed with a strong cation exchange resin (Lewatit S 100 MB/H strong acid, Bayer AG Leverkusen, Germany) and analysed for free amines prior to use by addition of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH) giving rise to a yellow colour (Dhbt-O- anion) if free amines are present. Solvent DCM (dichloromethane, analytical grade, Riedel de-Häen, Germany) was used directly without purification. Solvent NMP (N-methylpyrrolidone, Riedel de-Häen) was used without purification.

Amino acids

Fmoc-protected amino acids and corresponding pentafluorophenyl (Pfp) esters were purchased from MilliGen (UK), NovaBiochem (Switzerland) and Bachem (Switzerland), and the Dhbt-esters from NovaBiochem (Switzerland) in suitable side-chain protected forms. Boc-protected amino acids were purchased from Bachem (Switzerland).

Coupling reagents

Coupling reagent diisopropylcarbodiimide (DIC) was purchased from Riedel de-Häen, Germany, and destilled prior to use, dicyclohexylcarbodiimide (DCC) was purchased from Merck-Schuchardt, München, Germany, and purified by destillation. O-benzotriazolyl-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU) was purchased from PerSeptive Biosystems GmbH Hamburg, Germany, and benzotriazolyl-N-oxy-pyrrolidinium phosphonium hexafluorophosphat (PyBOP) and 7-azabenzotriazolyl-N-oxy-pyrrolidinium phosphonium

hexafluorophosphat (PyAOP) from PerSeptive.

Linker

Linker 4-hydroxymethylphenoxyacetic acid (HMPA, Novabiochem,
5   Switzerland) was coupled to the resin if required as a preformed
1-hydroxybenzotriazole (HObt) ester generated by means of DIC.

Solid phase

Peptides synthesised according to the Fmoc-strategy were
10   synthesised on three different types of solid support using 0.05
M or higher concentrations of Fmoc-protected activated amino acid
in DMF. 1) PEG-PS (polyethyleneglycol grafted on polystyrene;
TentaGel S NH2 resin, 0.27
mmol/g, TentaGel S RAM resin (s = 0.23 mmol/g), Rapp Polymere,
15   Germany or NovaSyn TG resin, 0.29 mmol/g (denoted s), Novabiochem,
Switzerland); 2) PepSyn K (Kieselguhr supported
polydimethylacrylamide resin functionalised with sarcosine methyl
ester 0.11 mmol/g; MilliGen, UK); 3) Chlorotrityl resin, 0.3 - 0.9
mmol/g (Novabiochem). In the two first cases the first amino acid
20   was coupled to the resin via linker HMPA except for TentaSel S RAM
which is coupled directly affording peptide amides. In the third
case the first amino acid was coupled directly to the chlorotrityl
resin. Peptides synthesised according to the Boc-strategy were
synthesised on a Merrifield-resin (polystyrene-divinylbenzene) with
25   the first amino acid attached (Novabiochem).

Catalysts and other reagents

Diisopropylethylamine (DIEA), was purchased from Aldrich, Germany,
ethylenediamine from Fluka, and piperidine from Riedel-de Häen,
30   Frankfurt, Germany. 4-(N,N-dimethylamino)pyridine (DMAP) was
purchased from Fluka, Switzerland and used as a catalyst in
coupling reactions involving symmetrical anhydrides. 3,4-dihydro-3-
hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH) was obtained from

Fluka, Switzerland, 1-hydroxybenzotriazole (HObt) from NovaBiochem, Switzerland and 1-hydroxy-7-azabenzotriazole

(HOAt) from PerSeptive. Biotin was purchased from Sigma. Scavengers Triisopropylsilan, ethanedithiol and thioanisol was

5   purchased from Aldrich, Germany.

General coupling procedures

The first amino acid was coupled as a symmetrical anhydride in DMF generated from the appropriate N-α-protected amino acid and DIC.

10   The following amino acids were coupled as Pfp- or Dhbt-esters or as preformed HOBt or HOAt esters made from appropriate N-α-protected amino acids and HObt or HOAt by means of DIC or TBTU in DMF unless otherwise stated.

15   ### Deprotection of the N-α-amino protecting group

Deprotection of the Fmoc group was performed by treatment with 20% piperidine in DMF (1x3 and 1x7 min) followed by wash with DMF until no yellow colour (Dhbt-O-) could be detected after addition of Dhbt-OH to the drained DMF. Deprotection of the Boc group was

20   performed by treatment with 50% TFA in DCM (v/v) 1x1.5 min and 1x20 min followed by washing 6x9 min each with DCM, neutralisation with 10% triethylamine in DCM (v/v) 2x1.5 min each, followed by 6x9 min washing with DCM.

25   ### Cleavage from solid support

Cleavage of peptide and N-terminal product from solid phase and simultaneous side-chain deprotection were done with acid. Peptides were cleaved from the resins by treatment with 95% trifluoroacetic acid (TFA, Halocarbon Products Corporation, USA; Biesterfeld & Co.

30   Hamburg, Germany)-water v/v or with TFA (trifluoroacetic acid)-H_2O-ethanedithiole-thioanisole (90:5:3:2 vol./vol./vol./vol.) or with TFA-TIS and other scavengers at room temperature for 2 h. The filtered resins were washed with 95% TFA-water and filtrates and

washings concentrated under reduced pressure. The residue was treated with ether and the precipitate freeze dried from acetic acid-water. The crude freeze dried product was analysed by HPLC and identified by MALDI-TOF MS.

5

Preformed symmetrical anhydride (PSA)

6 eq. N-α-amino protected amino acid was dissolved in DCM and cooled to 0°C. DCC (3 eq.) was added and the reaction continued for 10 min to yield PSA. The solvent was removed in vacuo and the remanens dissolved in DMF. The solution was filtered and immediately added to the resin followed by 0.1 eq. of DMAP.

Estimation of the coupling yield of the first N-α-amino protected amino acid

15 3-5 mg dry Fmoc-protected peptide-resin was treated with 5 ml 20% piperidine in DMF for 10 min at room temperature and the UV absorption for the dibenzofulvene-piperidine adduct was estimated at 301 nm. The yield was determined using a calculated extension coefficient e301 based on a Fmoc-Ala-OH standard.

20

In case of Boc-protection, the coupling was estimated according to the ninhydrin-method after removal of the Boc-group (Sarin et al., 1981, ref. 7).

25   Peptide synthesis on PepSyn K resin

Dry PepSyn K (ca 500 mg), was covered by ethylenediamine and left at room temperature over night. The resin was drained and washed with DMF 10 x 15 ml, 5 min each. After draining, the resin was washed with 10% DIEA in DMF v/v (2x15 ml, 5 min each) and finally washed with DMF until no yellow colour could be detected by addition of Dhbt-OH to the drained DMF. 3 eq. HMPA 3 eq. HObt and 3 eq. DIC was dissolved in 10 ml DMF and left for activation for 10 min, after which the mixture was added to the resin and the

coupling continued for 24 h. The resin was drained and washed with DMF (10x15 ml, 5 min each), and the acylation was checked by the ninhydrin test. The first amino acid was coupled as the preformed symmetrical anhydride (see above), and the coupling yields

5 estimated as described above. It was in all cases better than 70%. The remaining amino acids according to the sequence were coupled as described above. The couplings were continued for 2 h unless otherwise specified. Excess reagent was then removed by DMF washing. After completed synthesis the peptide-resin was washed

10 with DMF and diethyl ether
and dried in vacuo.

Peptide synthesis on PEG-PS

TentaGel S NH2 or NovaSyn TG resin (250 mg, 0.27-0.29 mmol/g) was

15 placed in a polyethylene vessel equipped with a polypropylene filter for filtration. The resin was swelled in DMF (5ml), and treated with 20% piperidine in DMF to secure the presence of non-protonated amino groups on the resin. The resin was drained and washed with DMF until no yellow colour could be detected after

20 addition of Dhbt-OH to the drained DMF. HMPA (3 eq.) was coupled as a preformed HObt-ester as described above and the coupling was continued for 24 h. The resin was drained and washed with DMF (5x5 ml, 5 min each) and the acylation checked by the ninhydrin test. The first amino acid was coupled as a preformed symmetrical

25 anhydride as described above. The coupling yields of the first Fmoc-protected amino acids were estimated as described above. It was in all cases better than 70%. The following amino acids according to the sequence were coupled as preformed Fmoc-protected HObt esters (3 eq.) as described above. The couplings were

30 continued for 2 h, unless otherwise specified. The resin was drained and washed with DMF (5x5 ml, 5 min each) in order to remove excess reagent. All acylations were checked by the ninhydrin test performed at 80 °C. After completed synthesis the peptide-resin was

washed with DMF (3x5 ml, 5 min each), DCM (3x5 ml, 1 min each) and finally diethyl ether (3x5 ml, 1 min each) and dried in vacuo.

Peptide synthesis on chlorotrityl resin

5   The chlorotrityl resin (approximately 0.8 mmol/g, NovaBiochem) and the first Fmoc-protected amino acid in the sequence was dried in vacuo overnight before use. Solvents, DCM and DMF, was dried over molecular sieves (4 Å, 24h) in a round bottomed flask for derivatisation dried at 100°C. The resin was placed in the flask

10   under argon and the Fmoc-amino acid (10 eq compared to the eq of resin) was dissolved in DCM (ca. 0.5 M) with a small amount of DMF added if necessary. The solution was added to the resin followed by addition of DIEA (20 eq) and the mixture left for 30 min with stirring. The resin was transferred to a syringe fitted with a

15   filter and washed with DCM/MeOH/DIEA (17:2:1) 3 times to cap remaining Cl, followed by washing with 3xDCM and 3xDMF. A small amount of resin, washed with DCM and MeOH prior to drying in a dessicator, was used for determination of substitution as described above by formation of dibenzofulvene-piperidine. However, treatment

20   with piperidine was extended to at least 3-4 h prior to analysis because of slow deprotection of the Fmoc-group. After deprotection of the Fmoc-group using a prolonged deprotection, the remaining amino acids according to the sequence were coupled as described above. The couplings were continued for 2 h unless otherwise

25   specified. Excess reagent was then removed by DMF washing. After completed synthesis the peptide-resin was washed with DMF and diethyl ether and dried in vacuo.

Fmoc-iminodiacetic acid (Fmoc-IDA)

30   The product was prepared by the following method: iminodiacetic acid (2.5 g, 0.0188 mol, Aldrich) was dissolved in 10% aqueous sodium carbonate, pH 9.5 (150 ml). Fmoc-Cl (4.85 g, 0.0188 mol, Bachem) was dissolved in tetrahydrofuran (50 ml) and added dropwise

over 30 min to the iminodiacetic acid solution. The mixture was left for 5 hr at room temperature, washed twice with ethyl acetate, acidified with aqueous HCl to pH 2 and Fmoc-iminodiacetic acid was extracted twice with ethylacetate. The combined extracts were

5   washed twice with aqueous HCl (pH 2) followed by washing with brine and then concentrated in vacuo. Pentane was added to turbidity and the crystallisation was allowed to proceed for 10 min on an ice bath. The Fmoc-iminodiacetic acid was collected by filtration and the filtrate washed with cold ethylacetate/pentane and dried to

10   give 5.61 g (84% yield). According to HPLC the purity was >99%. Mp. 212-214 °C. Literature. 213 - 215.5 °C.

N-(Fluoren-9-ylmethoxycarbonyl)-2-aminomalonic acid (Fmoc-2-AMA)

15   Diethyl 2-aminomalonate (Sigma, 200 mg, 0.95 mmol) was dissolved in an aqueous solution of sodium carbonate (10%, 6 ml) and the mixture refluxed and stirred for 3h after which TLC (EtOH:AcOH (4:1) and development with ninhydrin) showed complete hydrolysis of the diethyl ester. To the reaction mixture was added 1.2 eq of

20   O-(Fluoren-9-ylmethoxycarbonyl)-hydroxysuccinimide (NovaBiochem, Fmoc-OSu, 380 mg, 1.13 mmol) dissolved in dioxane (2 ml) over a period of 15 min and the solution stirred at room temperature overnight. Upon dilution with dioxane, the reaction mixture was acidified with HCl (1 M) to pH 5 and extracted with

25   dichloromethane. The aqueous phase was further acidified with HCl (1 M) to pH 2 upon which the product precipitated. The aqueous phase was added ethyl acetate and stirred for 10 min. prior to extraction with ethyl acetate. The organic phase was washed with water, dried over $MgSO_4$ and concentrated in vacuo. The product, was

30   crystallised from ethyl acetate - heptane with an overall yield of 135 mg (42%), mp. approximately 164°C, decomp. FAB-MS: Found 342; calculated 341.3. RP-HPLC analysis, tR 19 min, using above described conditions, revealed a minimal content of the

decarboxylated product, Fmoc-Gly-OH(< 3%).

N-(Fluoren-9-ylmethoxycarbonyl)-3-aminoglutaric acid (Fmoc-3-AGA)

5   3-Aminoglutaric acid (Sigma, β-glutamic acid, 30 mg, 0.20 mmol) was dissolved in 10% sodium carbonate/dioxane (1 ml/0.5 ml) and added Fmoc-OSu (83 mg, 0.25 mmol) dissolved in dioxane (1 ml) over a period of 15 min. After stirring at room temperature overnight, the reaction mixture was diluted with water and concentrated in vacuo

10   to remove dioxane prior to extraction with DCM. The aqueous phase was separated, acidified with HCl (1 M) to pH 2-3 and the precipitated product extracted into ethyl acetate. The organic phase was washed with water, dried over $MgSO_4$ and concentrated in vacuo. The product was crystallised from dioxane-water yielding 47

15   mg (63%), mp. 189-190 °C, RP-HPLC analysis, tR 21 min using above described conditions. FAB-MS: Found 370; calculated 369.4

Allyloxycarbonyl-3-aminoglutaric acid (Aloc-3-AGA)

3-Aminoglutaric acid (107 mg, 0.73 mmol) was dissolved in H_2O-

20   dioxan (2.5 ml/2 ml). Triethylamine (0.2 ml, 2 eq.) and diallyl pyrocarbonate (0.12 ml, 1 eq., Lancaster, Mühlheim am Main, Germany) was added and the mixture heated under reflux over night. After evaporation of dioxan in vacuo the mixture was acidified with citric acid (10%, 3 ml) and extracted with ethyl acetate (3×5 ml).

25   The organic phase was washed with water, dried over $MgSO_4$ and finally concentrated in vacuo. Trituration with pentane yielded 76 mg (45%). M.p. 117-118°C. FAB-MS: Calc. for $C_9H_{13}NO_6$, 231.2 found 232.0.

30   **1-(4,4-Dimethyl-2,6-dioxo-cyclohex-1-ylidene)ethyl-β-Ala (Dde-β-Ala)**

β-Ala (100 mg, 1.12 mmol) was dissolved in EtOH (98%, 8 ml). 2-Acetyldimedone (614 mg, 3.36 mmol, NovaBiochem) and triethylamine

(0.24 ml, 3.36 mmol) was added and the mixture was refluxed for 6 hours. After continued stirring over night, the mixture was concentrated to give an oil. Dissolution in ethyl acetate (5 ml) was followed by neutralisation with citric acid (10%, 3x5 ml). The

5   organic phase was washed with water, dried over $MgSO_4$ and finally concentrated *in vacuo*. Re-crystallisation from the ethyl acetate gave 180 mg (63%). M.p. 213-214°C. FAB-MS: Calc. for $C_{13}H_{19}NO_4$, 253.3 found 254.0.

10   EXAMPLE 1

Seq ID 1

Synthesis   of   FmocN(CH$_2$CO-ProValValAlaGluSerProLysLysPro)$_2$-
Seq ID 1
chlorotrityl resin and FmocN(CH$_2$CO-ProValValAlaGluSerProLysLysPro-
OH)$_2$ (IDA-synthesis of Seq. ID 1)

15

Product: FmocN(CH$_2$CO-ProValValAlaGluSerProLysLysPro-OH)$_2$
also denoted FmocN(CH$_2$CO-Seq. ID 1-OH)$_2$ [LPA-I]

The antigenic C-terminal sequence of OspC from Borrelia burgdorferi

20   ProValValAlaGluSerProLysLysPro (Seq. ID 1) was assembled on chlorotrityl resin (s = 0.8 mmol/g) as described above and tested
Seq ID 1
for homogeneity. The peptide moiety ProValValAlaGluSerProLysLysPro has a molecular weight of 1035 Dalton and a corrected substitution of 0.44 is used for samples of the peptide-resin. 100 mg of the

25   peptide-resin was suspended in DMF and deprotected with 20% piperidine-DMF. 0.55 eq Fmoc-iminodiacetic acid (i.e. 10% excess; 0.1x1.1x05x0.44x355 = 8.6 mg; Mw 355), 3.3 eq TBTU (0.1x1.1x3x0.44x321 = 46.6 mg; i.e. 3 times excess compared to the amount carboxy groups on Fmoc-iminodiacetic acid), 3.3 eq HObt

30   (0.1x1.1x3x0.44x135 = 19.6) and 4.95 eq DIEA (0.1x1.1x4.5x0.44x174 = 38 ml; standard amount of DIEA is 1.5xTBTU-eq.) was added to the resin and left overnight. Excess reagents were removed, the resin was washed with DMF, followed by ether and dried. The resulting

dimeric compound, FmocN(CH₂CO-ProValValAlaGluSerProLysLysPro-OH)₂,

Seq ID 1

was cleaved from the resin with 95% aqueous TFA. MALDI TOF MS: Calculated for $C_{115}H_{177}N_{25}O_{32}$, 2421.8; found 2423.2. The product contains only a small amount of the side-product FmocN(CH₂CO-

Seq ID 1

5   ProValValAlaGluSerProLysLysPro-OH)CH₂COOH   (Calculated for $C_{67}H_{97}N_{13}O_{19}$, 1388.6; found 1389.0) which is essentially absent upon repeated coupling.

EXAMPLE 2

10

Seq ID 1

Synthesis   of   biotin-NH(CH₂)₅CON(CH₂CO-ProValValAlaGluSer-ProLysLysPro-OH)₂ (IDA derivative of Seq. ID 1 extended with 6-amino-hexanoic acid and biotinylated)

15   Product: biotin-NH(CH₂)₅CON(CH₂CO-ProValValAlaGluSerProLysLysPro-OH)₂ also denoted Biotin-NH(CH₂)₅CON(CH₂CO-Seq. ID 1-OH)₂ [LPA-II]

Seq ID 1

FmocN(CH₂CO-ProValValAlaGluSerProLysLysPro)₂-chlorotrityl resin was deprotected with 20% piperidine-DMF and coupled with Fmoc-6-amino-

20   hexanoic acid activated with TBTU. The coupling was followed by exhaustive acetylation with acetic anhydride. After deprotection with 20% piperidine-DMF, biotin ($C_{10}H_{16}N_2O_3S$) was coupled, activated with TBTU. The product was cleaved from the resin with 95% aqueous TFA. MALDI TOF MS analysis showed the target compound as the major

25   product (calculated for $C_{116}H_{192}N_{28}O_{33}S$, 2539.0; found 2536.3)

Seq ID 1

containing some side-product, biotin-NH(CH₂)₅CON(CH₂CO-ProValAla-GluSerProLysLysPro-OH)CH₂COOH (calculated for $C_{68}H_{112}N_{16}O_{20}S$, 1505.8; found 1504.1).

30   EXAMPLE 3

Seq ID 1

Synthesis   of   FmocNHCH(CH₂CO-ProValValAlaGluSerProLysLysPro)₂-

Seq ID 1

chlorotrityl resin and NH₂CH(CH₂CO-ProValValAlaGluSerProLysLysPro-

OH)₂ (AGA-3-derivative of Seq. ID 1)

Product: NH₂CH(CH₂CO-ProValValAlaGluSerProLysLysPro-OH)₂

also denoted NH₂CH(CH₂CO-Seq. ID 1-OH)₂ [LPA-III]

5

The resin derivative was assembled in the same way as described
above for the iminodiacetic acid product using N-(Fluoren-9-
ylmethoxycarbonyl)-3-aminoglutaric acid. The resulting Fmoc-
NHCH(CH₂CO-ProValValAlaGluSerProLysLysPro)₂-chlorotrityl resin was   *Seq ID 1*

10   deprotected with 20% piperidine-DMF and NH₂CH(CH₂CO-ProValValAlaGlu-   *Seq ID 1*
SerProLysLysPro-OH)₂ was obtained by cleavage from the resin with
95% aqueous TFA. MALDI-TOF MS: Calculated for C₁₀₁H₁₆₉N₂₅O₃₀, 2213.6;
found 2215.2 (Figures 1 and 2). The higher molecular ions are the
sodium (2238.0) and potassium ion adducts (2254.3) respectively.

15   The product contains only a very small amount of lower molecular
weight products as evidenced from the spectra. HPLC of crude H-   *Seq ID 1*
ProValValAlaGluSerProLysLysPro-OH and crude NH₂CH(CH₂CO-ProValVal-   *Seq ID 1*
AlaGluSerProLysLysPro-OH)₂, is shown in Figures 3 and 4,
respectively. The HPLC of the HPLC-purified LPA-III is shown in

20   Figure 5.

EXAMPLE 4

Synthesis of Fmoc-Lys(Fmoc)-NHCH(CH₂CO-ProValValAlaGluSerPro-   *Seq ID 1*

25   LysLysPro)₂-chlorotrityl resin and H-Lys-NHCH(CH₂CO-Pro-   *Seq ID 1*
ValValAlaGluSerProLysLysPro-OH)₂ (AGA-3-derivative of Seq. ID 1
extended with di-Fmoc-lysine)

Product: H-Lys-NHCH(CH₂CO-ProValValAlaGluSerProLysLysPro-OH)₂ also

30   denoted H-Lys-NHCH(CH₂CO-Seq. ID 1-OH)₂ [LPA-IV]

   Seq ID 1

The above synthesised NH₂CH(CH₂CO-ProValValAlaGluSerProLysLysPro)₂-
chlorotrityl resin was coupled with di-Fmoc-lysine in 3 times

excess with TBTU as coupling agent. After Fmoc-deprotection with 20% piperidine-DMF, H-Lys-NHCH(CH$_2$CO-

Seq ID 1

ProValValAlaGluSerProLysLysPro-OH)$_2$ was cleaved from the resin with 95% aqueous TFA and precipitated with ether. MALDI TOF MS analysis:

5   Calculated for C$_{107}$H$_{181}$N$_{27}$O$_{31}$, 2341.8; found 2343.2 (Figure 6).

EXAMPLE 5

Seq ID 13

Synthesis of (HO-ProLysLysProSerGluAlaValValPro-COCH$_2$)$_2$CH-NH-Lys-

Seq ID 2

10   (GlnLeuAlaAsnAsnLeuGluThrAlaThrAlaAspTrpLysGlnGlnValGlyGlnTyr-

H)$_2$(AGA-3-derivative of Seq. ID 1 extended with bis-ESAT-6, 51-70

13

sequence (Seq. ID 2), on lysine)

Seq ID 13

Product   (HO-ProLysLysProSerGluAlaValValPro-COCH$_2$)$_2$CH-NH-Lys-

Seq ID 2

15   (GlnLeuAlaAsnAsnLeuGluThrAlaThrAlaAspTrpLysGlnGlnValGlyGlnTyr-H)$_2$

also denoted (HO-Seq. ID 1-COCH$_2$)$_2$CH-NH-Lys(Seq. ID 2-H)$_2$ [LPA-V]

Seq ID 1

FmocNCH(CH$_2$CO-ProValValAlaGluSerProLysLysPro)$_2$-chlorotrityl resin

(55 mg; s = 0.44), was deprotected with 20% piperidine-DMF and

20   coupled with Fmoc-Lys(Fmoc)-OH activated with TBTU to give the

Seq ID 1

above Fmoc-Lys(Fmoc)-NHCH(CH$_2$CO-ProValValAlaGluSerProLysLysPro)$_2$-

chlorotrityl resin. After deprotection of Fmoc-groups the amino

acids according to the sequence (ESAT-6, 51-70) were coupled as the

Fmoc protected amino acids using HOAt and DIC as coupling agents

25   in 4 times excess with ≥2 h couplings followed by a double coupling

of ≥30 min. After completed assembly of the peptide chains, the

peptide-resin was washed with DMF and diethyl ether and dried in

vacuo. The assembled product was cleaved from the resin with 95%

TFA and 5% triisopropylsilane and precipitated by addition of

30   ether, redissolved in acetic acid/water and lyophilised to give 2

mg of the target product C$_{305}$H$_{481}$N$_{83}$O$_{97}$, (Mw 6862.7). HPLC showed a

major peak. The product was analysed for homogeneity by amino acid

analysis. Alanine and leucine was used for comparison giving 3941

and 1937 pmol respectively corresponding to a Ala/Leu ratio of 2.04 (exp. 2.00). A double analysis similarly gave 2.03. The product was analysed by N-terminal amino acid sequencing and all 20 amino acids according to the ESAT sequence (Seq. ID 2) were recorded. The

5 Borrelia OspC sequence (Seq. ID 1) is N-terminally coupled and consequently not sequenced.

EXAMPLE 6

Seq ID 13

10 Synthesis of *Seq ID 3* (HO-ProLysLysProSerGluAlaValValPro-COCH$_2$)$_2$N-Lys(AlaSerAlaAlaAlaGluIleGlyAlaPheAsnTrpGlnGlnGluThrMet-H)$_2$ (IDA-derivative of Seq. ID 13 extended with Seq. ID 3 on lysine)

Seq ID 13

Product: (HO-ProLysLysProSerGluAlaValValPro-COCH$_2$)$_2$N-Lys-

15 (AlaSerAlaAlaAlaGluIleGlyAlaPheAsnTrpGlnGlnGluThrMet-H)$_2$ *Seq ID 3* also denoted (HO-Seq. ID 13-COCH$_2$)$_2$N-Lys(Seq. ID 3-H)$_2$ [LPA-VI]

Seq ID 1

FmocN(CH$_2$CO-ProValValAlaGluSerProLysLysPro)$_2$-chlorotrityl resin (55

mg; s = 0.44), was deprotected with 20% piperidine-DMF and coupled

20 with Fmoc-Lys(Fmoc)-OH activated with TBTU to give Fmoc-Lys(Fmoc)- *Seq ID 1* N(CH$_2$CO-ProValValAlaGluSerProLysLysPro)$_2$-chlorotrityl resin. After deprotection of Fmoc-groups with 20% piperidine-DMF the amino acids according to the ESAT-6, 1-17 sequence were coupled as the Fmoc protected amino acids using HOAt and DIC as coupling agents in 4

25 times excess with ≥2 h couplings followed by a double coupling of ≥30 min. After completed assembly of the peptide chains, the peptide-resin was washed with DMF and diethyl ether and dried in vacuo. The product was cleaved from the resin with 95% TFA and 5% triisopropylsilane and precipitated by addition of ether,

30 redissolved in acetic acid/water and lyophilised to give 8.7 mg of the target product. MALDI TOF MS analysis: Calculated for C$_{264}$H$_{409}$N$_{69}$O$_{83}$S$_2$, 5941.7; found 5944.7 The product was analysed for homogeneity by amino acid analysis. Proline and valine from the

Borrelia pepC10 sequence was used for comparison with Gly from the ESAT-6, 1-17 sequence. Amino acid analysis gave 5044, 2823 and 1408 pmol for Pro, Val and Gly respectively giving a Gly/Pro ratio of 0.28 (exp. 0.33) and a Gly/Val ratio of 0.50 (exp. 0.500). A double

5   analysis similarly gave 0.27 and 0.55. The product was analysed by N-terminal amino acid sequencing and all 17 amino acids according to the ESAT-6, 1-17 sequence (Seq. ID 3) were recorded. The Borrelia OspC sequence (Seq. ID 1) is N-terminally coupled and consequently not sequenced.

10

EXAMPLE 7

Seq ID 4

Synthesis of CH₂(CH₂CO-β-Ala-β-AlaLysGluProAsnLysGlyValAsnPro-AspGluValβAla)₂, (Glutaric acid-derivative, Fmoc-β-Ala and Dde-β-

15   **Ala synthesis of Seq. ID 4)**

Seq ID 4

Product: CH₂(CH₂CO-β-Ala-β-AlaLysGluProAsnLysGlyValAsnPro-AspGluValβAla)₂ also denoted CH₂(CH₂CO-β-Ala-β-Ala-Seq. ID 4-β-Ala)₂ [LPA-VII]

20

Seq ID 4

The β-Ala-β-AlaLysGluProAsnLysGlyValAsnProAspGluValβ-Ala sequence
Seq ID 4
(LysGluProAsnLysGlyValAsnProAspGluVal of *Chlamydia trachomatis* DnaK 357-368) was assembled on chlorotrityl resin and on TentaGel S NH₂ resin with HMPA linker using standard coupling procedures with

25   PyAOP 1.1 eq. and DIEA 2.2. eq. as coupling agents in DMF. In both cases the N-terminal Fmoc-α-amino group was deprotected with piperidine-DMF and subsequently coupled with a mixture of Fmoc-β-Ala and Dde-β-Ala to give an orthogonal 1:1 Fmoc/Dde-protection of the N-terminus resin-peptide chains. This was achieved by coupling

30   with 0.75 eq. Fmoc-β-Ala and 0.25 eq. Dde-β-Ala using 1.1 eq. PyAOP and 2.2 eq. DIEA in NMP. The Fmoc-protecting group was deprotected with 20% piperidine-DMF followed by coupling with excess glutaric acid (6 eq.) and DIEA (12 eq.) in NMP over night. The remaining N-

terminal Dde-α-protecting groups were deprotected with 2% hydrazine in NMP for 10 minutes, followed by washing with 5% DIEA in NMP. Assembly was then achieved over night by activation with 6 eq. PyAOP and 12 eq. DIEA in NMP. The resulting product was cleaved

5   from the resin with TFA-H$_2$O-TIS (92.5:5:2.5, vol/vol/vol). A single product with the correct mass was obtained with both resins. MALDI TOF MS: Calc. for C$_{135}$H$_{218}$N$_{38}$O$_{50}$, 3173.4; found 3169.7. (trityl-resin). Figure 9 shows the MALDI-TOF MS spectrum of the product.

10   EXAMPLE 8

Seq ID 4

Synthesis of HC(CH$_2$CO-LysGluProAsnLysGlyValAsnProAspGluValβAla)$_2$COOH

(TCA-derivative, CH(CH$_2$COOH)$_2$COOH synthesis of Seq. ID 4)

Seq ID 4

15   Product: HC(CH$_2$CO-LysGluProAsnLysGlyValAsnProAspGluValβAla)$_2$COOH also denoted HC(CH$_2$CO-Seq. ID 4-βAla)$_2$COOH [LPA-VIII]

Seq ID 4

The LysGluProAsnLysGlyValAsnProAspGluValβAla sequence was assembled on TentaGel S NH$_2$ resin with HMPA linker (s = 0.19 mmol/g; 0.02 g)

20   using standard coupling procedures with 3 eq. DIC and 3 eq. HOAt as coupling agents in DMF. The N-terminal Fmoc-α-amino group was deprotected with piperidine-DMF and coupled with 0.55 eq. of CH(CH$_2$COOH)$_2$COOH, 1.65 eq. PyAOP and 3.3 eq. DIEA in DMF over night. The resulting product was cleaved from the resin with TFA-H$_2$O-TIS-

25   EDT (90:5:2.5:2.5, vol/vol/vol/vol) and submitted to gel filtration on G-25 Sephadex with 1% AcOH as eluent. The resulting fractions were lyophilysed to give 2 mg of the pure main product, HC(CH$_2$CO-
Seq ID 4
LysGluProAsnLysGlyValAsnProAspGluValβAla)$_2$COOH (approx. 20% yield). MALDI TOF MS: Calc. for C$_{124}$H$_{198}$N$_{34}$O$_{48}$, 2933.1; found 2932.6. Figure

30   10 shows the MALDI-TOF MS spectrum for the product, and Figure 11 shows the HPLC chromatogram.

EXAMPLE 9

Seq ID 5

Synthesis of Fmoc-NHCH(CH₂CO-AspArgValTyrIleHisProPheHisLeu-NH₂)₂, (AGA-3-derivative of angiotensin-I amide, Fmoc-3-AGA synthesis of Seq. ID 5)

Seq ID 5

5   Product: Fmoc-NHCH(CH₂CO-AspArgValTyrIleHisProPheHisLeu-NH₂)₂ also denoted Fmoc-NHCH-(CH₂CO-Seq. ID 5-NH₂)₂ [LPA-IX]

Seq ID 5

The peptide sequence AspArgValTyrIleHisProPheHisLeu was assembled on TentaGel S RAM resin using standard coupling procedures with DIC

10   and HOAt as coupling agent in DMF. The N-terminal Fmoc-α-amino group was deprotected with piperidine-DMF and coupled with 0.55 eq. Fmoc-AGA, 3.3 eqv TBTU, 3.3 eqv. HOBt and 4.95 eqv. DIEA in NMP over night. The assembled product was cleaved from the resin with TFA-H₂O-triisopropylsilane (90:5:5, vol/vol/vol) to give Fmoc-

Seq ID 5

15   NHCH(CH₂CO-AspArgValTyrIleHisProPheHisLeu-NH₂)₂ as the major product according to MS. MALDI TOF MS: Calc. for $C_{144}H_{195}N_{37}O_{30}$, 2924.4; found 2923.6.

EXAMPLE 10

20

Seq ID 5

Synthesis of Fmoc-NHCH(CH₂CO-AspArgValTyrIleHisProPheHisLeu-NH₂)₂ (AGA-3-derivative of angiotensin-I amide, Fmoc-3-AGA synthesis of Seq. ID 5)

Seq ID 5

25   Product: Fmoc-NHCH(CH₂CO-AspArgValTyrIleHisProPheHisLeu-NH₂)₂ also denoted Fmoc-NHCH(CH₂CO-Seq. ID 5-NH₂)₂ [LPA-IX]

Seq ID 5

The peptide sequence AspArgValTyrIleHisProPheHisLeu was assembled on TentaGel S RAM resin (AM-linker) using standard coupling

30   procedures with 3 eq. DIC and 3 eq. HOAt as coupling agent in DMF. The N-terminal Fmoc-α-amino group was deprotected with piperidine-DMF and coupled with 0.55 Fmoc-AGA, 3 eqv. PyAOP, 6 eqv. DIEA in DCM/NMP (3:1 vol/vol) dissolving Fmoc-AGA in NMP, PyAOP (activating

agent) in DCM and adding DIEA. Coupling over night. The assembled product was cleaved from the resin with TFA-H$_2$O-triisopropylsilane (90:5:5, vol/vol/vol) to give Fmoc-NHCH(CH$_2$CO-AspArgValTyrIleHis- _Seq ID 5_ ProPheHisLeu-NH$_2$)$_2$ as the major product according to MS. MALDI TOF

5   MS: Calc. for C$_{144}$H$_{195}$N$_{37}$O$_{30}$, 2924.4; found 2920.8. The product may be further purified by preparative HPLC or Gel filtration if desired. Figure 12 shows the MALDI-TOF MS spectrum for the crude product. Figure 13 shows the HPLC chromatogram for the crude product.

10

EXAMPLE 11

Seq ID 5

Synthesis of Aloc-NHCH(CH$_2$CO-AspArgValTyrIleHisProPheHisLeu-NH$_2$)$_2$ (AGA-3-derivative of angiotensin-I amide, Fmoc-β-Ala and Dde-β-Ala

15 synthesis of Seq. ID 5 using orthogonal protection of the sequence to be assembled)

Seq ID 5

Product: Aloc-NHCH(CH$_2$CO-AspArgValTyrIleHisProPheHisLeu-NH$_2$)$_2$ also denoted Aloc-NHCH-(CH$_2$CO-Seq. ID 5-NH$_2$)$_2$ [LPA-X].

20

Seq ID 5

The peptide sequence AspArgValTyrIleHisProPheHisLeu was assembled on TentaGel S RAM resin using standard coupling procedures with 3 eq DIC and 3 eq. HOAt as coupling agents in DMF. The N-terminal Fmoc-α-amino group was deprotected with piperidine-DMF and

25 subsequently coupled with a mixture of Fmoc-β-Ala and Dde-β-Ala to give an orthogonal 1:1 Fmoc/Dde-protection of the N-terminus resin-peptide chains. The Fmoc-protecting group was deprotected with 20% piperidine-DMF followed by over night coupling with excess Aloc-3-AGA (6 eq.) and DIEA (12 eq.) in NMP. The remaining N-terminal Dde-

30 α-amino groups were deprotected with 2% hydrazine in NMP for 10 minutes, followed by washing with 5% DIEA in NMP. Assembly was then achieved over night by activation with 6 eq. PyAOP and 12 eq. DIEA in NMP. The resulting product was cleaved from the resin with TFA-

H_2O-TIS (92.5:5:2.5, vol/vol/vol). MALDI TOF MS: Calc. for $C_{139}H_{199}N_{39}O_{32}$, 2928.6; found 2927.5.

EXAMPLE 12

5

Synthesis of Fmoc-AspProThrGlnAsnIleProProGly-NHCH(CH₂CO- *Seq. ID 6* AspArgValTyrIleHisProPheHisLeu-NH₂)₂ *Seq. ID 5* (AGA-3-derivative of angiotensin-I amide extended with Chlostridium thermosacchrolyticum peptide sequence 19-27)

10

Product: Fmoc-AspProThrGlnAsnIleProProGly-NHCH(CH₂CO-AspArg- *Seq. ID 6* ValTyrIleHisProPheHisLeu-NH₂)₂ *Seq. ID 5* also denoted Fmoc-Seq. ID 6-NHCH(CH₂CO-Seq. ID 5-NH₂)₂ [LPA-XI]

15 The Chlostridium thermosaccharolyticum peptide sequence 19-27 AspProThrGlnAsnIleProProGly was synthesised on chlorotrityl resin *Seq. ID 6* using standard peptide synthesis and cleaved from the resin with TFA-DCM (1:99, vol/vol) affording the fragment Fmoc-Asp(tBu)ProThr-(tBu)Gln(Trt)Asn(Trt)IleProProGly-OH. FmocNHCH(CH₂CO-AspArgValTyr- *Seq. ID 11* *Seq. ID 5*

20 IleHisProPheHisLeu-NH₂)₂ was assembled as above (Example 11) and coupled with 3 eq. of the fragment using 3 eq. PyAOP and 6 eq. DIEA as coupling agents in NMP over night. The resulting fragment coupled product was cleaved from the resin with TFA-H2O-TIS (90:5:5, vol/vol/vol) to give the target compound together with *Seq. ID 5*

25 some NH₂CH(CH₂CO-AspArgValTyrIleHisProPheHisLeu-NH₂)₂. MALDI TOF MS: Calc. for $C_{184}H_{254}N_{46}O_{46}$, 3846.3; found 3842.8.

EXAMPLE 13

30 The performance of an ELISA based on LPA-I.

The capability of LPA-I according to the present invention to react with sera from patients with neuroborreliosis was evaluated against that of an ELISA based on biotinylated-pepC10, wherein pepC10 is

identical to the amino acid sequence of Seq. ID 1.

Indirect IgM-ELISA using LPA-I and biotinylated-pepC10

Preparing plates for the LPA-I-ELISA: Microtiter plates (Maxisorb;
Nunc, Roskilde, Denmark) were coated overnight at 4°C with 0.6
µg/ml of LPA-I diluted in 0.05 M bicarbonate pH 9.6, blocked with
3% (w/v) milk powder in phosphate-buffered saline (PBS) for 1 h,
and reacted with sera diluted 1/200 in 1% (w/v) milk powder in PBS
containing 0.05% Tween 20 (PBST) and 0.7 M NaCl for 2 h. The
secondary antibody was a peroxidase conjugated rabbit anti-human
IgM (Dako, Denmark, codes P-215) diluted 1/1000 in 1% (w/v) milk
powder in PBST. After 1 h of incubation, bound secondary antibody
was quantitated by colouring with o-phenylenediamine and H_2O_2 in
citrate buffer (Sigma, St. Louis, Mo.) for 30 min. Optical density
(OD) at 492 nm was determined in a plate reader (EAR 400 AC-SLT,
Labinstruments, Austria). Plates were washed extensively with PBST-
0.5M NaCl between each incubation step. The 98% specific diagnostic
cut-off level was OD of 0.270 for IgM detection.

Preparing plates for the biotinylated-pepC10-ELISA: ELISAs with
biotinylated-pepC10, were performed essentially as described above
for LPA-I, except that microtiter plates were first coated with
streptavidin (2.5 µg/ml) (Zymed, S. Avidinii) in citrate buffer (pH
5) overnight at 4°C, and then incubated with biotinylated pepC10
(0.1 µg/ml) in 1% (w/v) milk powder in PBST-0.37 M NaCl overnight
at 4°C.

Serum specimens from patients

(i) Sera from 97 Danish patients with neuroborreliosis (NB).
All patients had been hospitalised in 1994 (58 males and 42 females
between 4 and 80 years of age; median age, 49). The selection was
based on a positive test for B. burgdorferi specific intrathecal
antibody synthesis. The diagnosis was further corroborated by the

presence of lymphocytic pleocytosis in CSF (median cell count, 124/μl) and one of the following manifestations: lymphocytic meningoradiculitis with typical painful radiculitis, mononeuritis multiplex (Bannwarth's syndrome), radiculomyelitis, monosymptomatic fascialis paresis (all children), subacute lymphocytic meningitis, and chronic progressive encephalomyelitis. Of the 100 patients, 48 recalled a previous EM-like skin lesion. Disease duration, defined as the time from onset of neurological symptoms until diagnostic blood and CSF samples were taken, ranged from 1 week to 26 weeks (median duration, 3 weeks).

Control serum specimens

Sera from 80 healthy controls were used for determination of the 98%-specific cut-off level in ELISAs.

Results

Comparing results of the LPA-I ELISA with results of the biotinylated-pepC10 ELISA.

The following table (Table 1) shows the frequency (%) of positive Lyme borreliosis sera in the early stages of Lyme borreliosis found by the above-described LPA-I and biotinylated-pepC10 ELISAs.

TABLE 1. IgM reactivity of NB sera to biotinylated-pepC10 and LPA-I

	No. (%) of IgM reactive sera to	
	biotinylated pepC10	LPA-I
NB (n=97)	n=42 (43.3%)	n=41 (42.3%)

It can be concluded that the two assays have nearly the same sensitivity.

Figure 7 compares the individual results obtained in patients with NB regarding the quantitative measurement of IgM in the two ELISAs. The vertical and horizontal lines marks the 98% specific diagnostic cut-off levels for the respective LPA-I and biotinylated-pepC10

5   ELISA (0.275 and 0.460). As can be seen the OD titres are of the same magnitude in both assays.

It can be concluded that in this set-up, the sensitivity of the two assays are comparable. Further since the OD cut-off-value in the

10   pepC10 ELISA is higher than the OD cut-off-value in the LPA-I ELISA it is expected that the LPA-I ELISA will perform better than the biotinylated-pepC10-ELISA when testing a large number of serum samples. Importantly, three serum samples in Figure 7 tested positive in the biotinylated-pepC10 ELISA but negative in the LPA-I

15   ELISA. The positive reaction of all three sera is not due to anti-Borrelia antibodies but is due to the presence of anti-streptavidin antibodies which reacted with streptavidin in the biotinylated-pepC10 ELISA only. The present invention, therefore, is superior to ELISA procedures which employ peptides coupled to streptavidin

20   because is reduces the number of false-positive serum samples.

EXAMPLE 14

Immunogenicity of the LPA-VI construct.

25   The capability of an LPA according to the present invention to induce a humoral immune response was studied by immunising mice with LPA-VI. Five female C57 black mice (age 6 to 8 weeks) were immunised two times intraperitoneally with a 2-week interval and were bled on days 0, 14, and 28. Each immunisation dose contained

30   5 μg of LPA-VI dissolved in 0.25 ml 0.9% NaCl and supplemented with 1 mg Al(OH)$_3$ (Superfos Biosector, Denmark) and 0.25 ml Freund's incomplete adjuvant. The serum samples were diluted 200 fold and then tested by ELISA for Ig reactivity against OspC produced on

recombinant form (WO 97/42221) and LPA-I (Figure 8). It is concluded that LPA-VI is highly effective in inducing antibodies against the C-terminal B-cell epitope of OspC since the mice generated antibodies which recognise both recombinant OspC and LPA-I.

Figure 8. Mean ELISA titer of sera from five mice immunised with LPA-VI on days 0 and 14. The microdilution plates were coated with LPA-I (diamond) or recombinant OspC (Squares), and the sera were analysed in a 200-fold dilution.

SEQUENCE LISTING

<110> HOLM, Arne and Statens Serum Institut

<120> Method for preparing and Ligand Presenting Assembly
(LPA), and LPA, and uses thereof

<130> P199801104 WO

<140>
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29. US 4 599 231

15   30. US 4 599 230

31. US 4 596 792

32. US 4 578 770

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